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Influence of Genotype Variations on Markers of Skeletal Muscle Recovery from Heavy
Endurance Exercise, and on Responses to Carbohydrate-Protein Supplementation

Qingnian Goh

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Science

Kinesiology

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Dedication

This thesis is dedicated to:

The James Madison University Department of Kinesiology graduate teaching assistants (class of 2010) for their much cherished friendship, and for creating an amazing working and learning environment,

The James Madison University Exercise Science faculty and staff (2008 – 2010) for their kind and valuable guidance in the past two years, as well as their continued faith and support throughout the undertaking of this study,

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Table of Contents

Dedication.....	ii
Acknowledgments.....	iii
List of Tables.....	v
List of Figures.....	vi
Abstract.....	vii
I. Introduction.....	1
II. Review of the Literature.....	6
III Methods.....	32
IV Manuscript.....	46
V Conclusion.....	88
Appendices.....	89
References	96

List of Tables

Table 2.1: Carbohydrate and Protein and Acute Endurance Performance.

Table 2.2: Carbohydrate and Protein and Post-Exercise Recovery.

Table 2.3: Genotypes and Sport Performance.

Table 2.4: Genotypes and Muscle Performance.

Table 2.5: Genotypes and skeletal muscle damage/recovery.

Table 3.1: TaqMan primer sets for SNPs tested.

Table 4.1: TaqMan primer sets for SNPs tested.

Table 4.2: Subject Demographics.

Table 4.3: Genotype Distributions and Frequencies.

Table 4.4: Subsequent Exercise Performance – 20 km Time Trial (CHO Treatment).

Table 4.5: Genotype and Subsequent Exercise Ride Times for CHO treatment.

Table 4.6: Treatment by Genotype interaction in Subsequent Exercise.

Table 4.7: Markers of Recovery for CHO Treatment.

Table 4.8: Significant Associations of Genotypes with Markers of Muscle Recovery.

Table 4.9: Treatment x time within Genotypes on markers of muscle recovery.

Table 4.10: Treatment x Time Effects on markers of muscle recovery within “susceptible” Genotype groups.

List of Figures

Figure 3.1: Timeline for exercise trials and measurements of recovery.

Figure 4.1: Timeline for exercise trials and measurements of recovery.

Abstract

Purpose: This study examined the influence of genotype polymorphisms (specifically ACTN3 R577X, IGF-II C13790C, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T) on markers of skeletal muscle recovery following an acute bout of heavy endurance exercise, and assessed their role in determining individual responses to carbohydrate-protein supplementation. **Methods:** Twelve trained male cyclists completed repeated cycling trials, each consisting of an initial session of glycogen-depleting exercise, followed four hours later by a >1 hr time-trial on a computerized cycle ergometer (VeloTron, Racermate, Inc). Subjects were randomly administered a high-carbohydrate low-protein (HCLP), or carbohydrate-only (CHO) beverage, which was consumed immediately and 2-hours post glycogen depleting exercise, and immediately following subsequent exercise. Beverages were isocaloric and isovolumetric. Blood samples were obtained before the glycogen-depleting exercise of the first trial for genotyping analysis. Serum creatine kinase (CK), peak muscle function (MVC), mental and physical energy and fatigue ratings, and muscle soreness ratings were obtained as markers of muscle recovery at specific time points. Subjects completed these methods for both treatments in a double-blind protocol. **Results:** Changes in MVC were significantly different ($P < 0.05$) from baseline to 4-hours post glycogen depleting exercise among genotypes for the ACTN3 R577X, IGF-II C13790C, and IGF2AS A1364C single nucleotide polymorphisms (SNPs) during the CHO control trial. Changes in mental/physical energy/fatigue and muscle soreness ratings were significantly different ($P < 0.05$) from baseline to 24-hours post glycogen depleting exercise among genotypes for the ACTN3 R577X and IGF2AS G11711C SNPs during the CHO control trial. These

variables tended to improve with the HCLP treatment when subjects identified for the above genotype variations were analyzed, although the improvements were not statistically significant. Changes in serum CK were not significantly different ($P > 0.05$) from baseline to 4-hours post glycogen depleting exercise among genotypes analyzed.

Conclusions: Genotype variations led to significant differences in MVC, physical/mental energy/fatigue and muscle soreness ratings, which tended to improve with the HCLP treatment. There were no genotype effects for serum CK.

Key Words: Genotype, Carbohydrate, Protein, Muscle Damage, Recovery

Chapter I

Introduction

This study shall attempt to analyze the roles of genotypes on the extent of muscle damage, and the subsequent rate of muscle recovery, following intense aerobic exercise. The findings should enhance understanding of the variations in response to post-exercise recovery beverages with protein among endurance-trained individuals.

Background

Prolonged high-intensity endurance exercise is associated with depleted glycogen stores and impairments in markers of muscle recovery. Traditionally, sports beverages containing water, carbohydrate, and electrolytes have been utilized by endurance athletes to enhance glycogen replenishment, facilitate muscle recovery, and improve aerobic performance (Hargreaves et al., 1984; Coyle and Coggan, 1984; Coggan and Coyle 1991; Coyle, 1992; 1992; Halson, 2004). Recently, the inclusion of protein into carbohydrate-based sport beverages to confer additional performance benefits has garnered considerable attention in the field of exercise science. The inclusion of protein content is proposed to enhance the rate of muscle recovery, as well as improve subsequent endurance performance (Saunders et al., 2004; Betts et al., 2007; Berardi et al., 2008; Rowland et al., 2008; and Skillen et al., 2008).

Current studies examining the efficacy of carbohydrate-protein supplementations have demonstrated mixed results. Some studies have documented improved performance in subsequent exercise bouts (Saunders et al., 2004; Betts et al., 2007; Berardi et al., 2008; Rowland et al., 2008; and Skillen et al., 2008), as well as reductions in markers of

muscle damage and soreness (Saunders et al., 2004; Millard-Stafford et al., 2005; Romano-Ely et al., 2006; Rowland et al., 2008; and Skillen et al., 2008) with carbohydrate-protein ingestion. However, other studies have reported no differences in subsequent exercise performances between carbohydrate and carbohydrate-protein beverages (Millard-Stafford et al., 2005; Romano-Ely et al., 2006; Betts et al., 2007). In addition to the variations between studies, there appears to be considerable variations among individual subjects with respect to the potential efficacy of carbohydrate-protein ingestion. For example, Combest et al. (2005) reported that 10 of 14 cyclists performing exhaustive exercise observed sizable attenuations in post-exercise serum CK levels ($\Delta\text{CK} = 975 \pm 590 \text{ U/L}$) with carbohydrate-protein ingestion, while the remaining four subjects were non-responders ($\Delta\text{CK} = -10.0 \pm 31.6 \text{ U/L}$) to carbohydrate-protein intake. Furthermore, the carbohydrate-protein ‘responders’ experienced improvements in subsequent exercise performance that were significantly greater than those who were ‘non-responders’.

The variations in findings within current literature suggest that some individuals are better responders to carbohydrate-protein supplementation than others, although the mechanism for the variations remains unclear. One possible explanation may lie in the role of specific genetic polymorphisms. Certain genotypes may predispose individuals to greater muscle damage following exercise. Hence, such individuals would potentially respond better to sports recovery beverages containing protein.

Rationale

Current studies examining genotype associations with muscle damage have identified specific genotypes associated with increased skeletal muscle damage following

acute exercise: MLCK C37885A (Clarkson et al., 2005), IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T (Devaney et al., 2007), and ACE ID (Yamin et al., 2007). However, these studies have primarily incorporated eccentric resistance exercise protocols. The association between genotypes and aerobic exercise-induced muscle damage is therefore not well understood.

Hence, the present study seeks to determine if genetic polymorphisms influence muscle damage/recovery variables following heavy endurance exercise. This would address and account for the wide variations in responses to performance recovery beverages containing protein following intense aerobic exercise.

Purpose

This study seeks to evaluate the influence of genetic polymorphisms (specifically IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T, and ACTN3 R577X) on the rate of muscle recovery, as measured by serum creatine kinase (CK) levels, peak muscle function (MVC), perceived muscle soreness, and ratings of energy/fatigue following heavy endurance exercise. The findings may help to determine the likelihood of individuals responding to recovery beverages containing protein.

Hypotheses

- 1) The variations between genotypes among subjects will lead to significant differences in the following muscle damage variables after acute heavy endurance exercise: serum CK levels, peak muscle function (MVC), perceived physical and mental energy and fatigue levels, and perceived muscle soreness levels.

- 2) Subjects with genotypes that are associated with greater increases in the above muscle damage variables will derive significant reductions in these variables, and significant improvements in time to completion during a subsequent endurance performance exercise with the ingestion of high carbohydrate – low protein beverages.

Assumptions

- 1) Subjects participated in a maximal cycle test before the start of the study to determine their VO_2 peak and maximal power. The oxygen uptake measurement served as an exclusion criterion, while the power score was used to determine workload performed during the glycogen depleting exercise, and the steady state portion of the subsequent exercise. Therefore, it was assumed that all subjects provided a maximal effort during this test.
- 2) Subjects completed a maximum voluntary isometric contraction of the quadriceps to assess peak muscle function on five separate occasions for each trial. It was assumed that subjects gave a maximal effort during each of these occasions.
- 3) It was assumed that subjects adhered to instructions to consume no other calories other than their standardized breakfast 12-hours prior to each training session. It was also assumed that subjects did not consume any calories besides the treatment provided during the 4-hour recovery period between the glycogen depleting exercise and the subsequent time-trial. It was finally assumed that subjects did not consume any calories besides the treatment provided for two hours after the subsequent time trial.

- 4) Subjects were instructed to treat each subsequent time-trial as a competitive race, and provide a maximal effort. It was therefore assumed that subjects provided a maximal effort during their subsequent performance rides.

Definition of Terms

- 1) Genotype: genetic constitution of an organism, determined by the allele composition.
- 2) Allele: Variations in DNA sequence in a chromosome (DNA and protein structure found in cells).
 - Heterozygous: possessing 2 different variants of an allele.
 - Homozygous: possessing identical alleles.
- 3) Polymorphism: Common variations of DNA resulting from mutations.
- 4) Single Nucleotide Polymorphism (SNP): Variation in DNA sequence between individuals involving a single nucleotide— A, G, C, T (nucleotides are molecules that form the structure of DNA).

Chapter II

Review of Literature

Carbohydrate-based sports beverages have been demonstrated to improve athletic performance during heavy endurance exercise. The addition of protein into carbohydrate-based sport beverages to confer additional performance benefits in aerobic exercise has been recently investigated within the last decade. Current literature is inconclusive on the effectiveness of carbohydrate-protein supplementation as performance enhancement beverages for acute endurance exercise (**Table 2.1**). Some studies have demonstrated enhanced cycling time trial performance and time to fatigue with carbohydrate-protein ingestion. Conversely, other studies reported a lack of substantial improvements in time to exhaustion and time to completion when protein calories were added into carbohydrate beverages.

When utilized as a performance recovery beverage, current literature is more positive on the effectiveness of carbohydrate-protein supplementation in subsequent endurance performance (**Table 2.2**), as evidenced by improvements in subsequent time trial performances and times to exhaustion. Studies examining physiological recovery variables have reported attenuated plasma CK levels, and reduced levels of perceived muscle soreness following heavy endurance exercise with carbohydrate-protein supplementation. Despite these documented benefits, there are variations between studies in the magnitude and extensiveness of the benefits among subjects. One possible explanation for the variability in recovery benefits with carbohydrate-protein supplementation, as well as the variations in findings on acute endurance exercise, may be the role of genotypes.

Genotyping studies have revealed that genetic variations may account for differences in sport performance (**Table 2.3**), muscle function (**Table 2.4**), and skeletal muscle damage accrued following exercise (**Table 2.5**). In sports performance, the ACTN3 R allele and RR genotype are associated with enhanced sprint performance, whereas the ACTN3 X allele and XX genotype are associated with augmented endurance performance. For muscle function, the ACE D allele and ACE DD genotype are associated with increased type II fiber distribution, and improved muscular strength, whereas the ACE I allele and ACE II genotype are associated with increased type I fiber distribution. In terms of skeletal muscle damage, MLCK C37885A, IGF II G/C, IGF2AS A1364C, IGF2AS G11711T, and ACE II allele have been identified with increased CK levels and/or strength loss following resistance exercise protocols.

The present study aims to examine the influence of genotypes on the rate of muscle recovery following heavy endurance exercise, which may help determine individual responses to recovery beverages containing protein.

Table 2.1: Carbohydrate and Protein and Acute Endurance Performance.

Articles	Problem/Question studied	Participants	Procedures	Findings
Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity (Ivy et al., 2003)	To compare the effects of a carbohydrate protein supplement against a carbohydrate-only supplement, and a sweetened placebo, on aerobic endurance performance.	9 trained male cyclists (27.3 ± 1.3 yr, 69.6 ± 2.5 kg, 52.6 ± 10.3 ml/kg/min)	Subjects performed three trials of cycle ergometry at 45 – 75% of $\text{VO}_{2\text{max}}$ for 3 hr, and then at 85% $\text{VO}_{2\text{max}}$ to exhaustion. During each separate trial, subjects were administered 200 ml of the sweetened placebo, a 7.75% liquid carbohydrate supplement, or a 7.75%/1.94% protein supplement immediately prior to the start of exercise, and every 20 minutes during exercise, until 85% $\text{VO}_{2\text{max}}$ was achieved. Subsequent trials were performed 7 – 14 days apart with the alternate treatment beverages.	The time to exhaustion was significantly longer with consumption of the carbohydrate-only supplement, when compared to the placebo. The time to exhaustion was significantly longer with consumption of the carbohydrate-protein supplement, when compared to the carbohydrate-only supplement. Measured physiological variables (respiratory exchange ratio, blood glucose, plasma insulin, plasma free fatty acid, and blood lactate) did not differ significantly between the carbohydrate-only and carbohydrate protein treatments. These findings suggest that the addition of protein content to a carbohydrate supplement improves aerobic endurance performance above that of a carbohydrate-only supplement, although the mechanism behind this benefit is not completely understood.
Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage (Saunders, Kane, and Todd, 2004)	To compare the effects of a carbohydrate-protein beverage against a carbohydrate-only beverage on cycling endurance performance and post-exercise muscle damage.	15 trained male cyclists (20.9 ± 3.3 yr, 73.3 ± 6.4 kg, 52.6 ± 10.3 ml/kg/min)	Subjects performed two trials of cycle ergometry to exhaustion (at 75% and 85% $\text{VO}_{2\text{peak}}$ respectively, 12 – 15 hr apart). Subjects were administered 1.8 ml/kg bodyweight of randomly assigned carbohydrate or carbohydrate-protein beverage every 15 minute during exercise, and 10 ml/kg bodyweight of the same treatment post-exercise. The protocol was repeated 7 – 14 days later with the other treatment beverage.	The time to exhaustion was significantly longer in the initial performance ride with consumption of the carbohydrate-protein beverage, when compared to the carbohydrate-only beverage ($P < 0.05$). This finding demonstrates a significant improvement in acute cycling times to exhaustion with the addition of protein calories in carbohydrate beverages.

Failure of protein to improve time trial performance when added to a sports drink (Van Essen and Gibala, 2006)	To compare the effects of adding 2% protein to a 6% carbohydrate beverage on 80 km cycling time trial performances against a 6% carbohydrate drink and a nonenergetic sweetened placebo.	10 trained male cyclists (24 ± 2 yr, 76 ± 2 kg, 63 ± 2 ml/kg/min)	Subjects performed three separate 80 km time trials on the cycle ergometer. During each separate trial, subjects were administered 250 ml of the carbohydrate-protein, carbohydrate, or placebo beverage every 15 minutes during exercise. Subsequent trials were performed 7 days apart with the alternate treatment beverages.	The times to completion were significantly faster with consumption of the carbohydrate-protein and carbohydrate beverages, than with consumption of the placebo ($P < 0.002$). There were no significant differences in time to completion between the carbohydrate-protein and carbohydrate beverages ($P = 0.92$). These findings suggest that while the carbohydrate-protein beverage improved 80 km cycle time trial performances, additional protein content does not appear to confer further benefits in improving the time to completion.
Effects of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance (Romano-Ely et al., 2006)	To compare the effects of a carbohydrate-protein-antioxidant beverage against a carbohydrate-only beverage on cycling endurance performance and post-exercise muscle damage.	14 trained male cyclists (24.0 ± 4.1 yr, 59.8 ± 11.9 ml/kg/min)	Subjects performed two trials of cycle ergometry to exhaustion (at 70% and 80% VO_2peak respectively, 24 hr apart). Subjects were administered 2.0 ml/kg bodyweight of randomly assigned carbohydrate-protein-antioxidant of carbohydrate-only beverage every 15 minutes during exercise, and 10 ml/kg bodyweight of the same treatment post-exercise. The protocol was repeated 1 – 2 weeks later with the alternate treatment beverage.	The times to fatigue were not significantly different between the two treatment beverages in the initial 70% VO_2peak trial ($P = 0.77$), and in the subsequent 80% VO_2peak trial ($P = 0.91$). Post-exercise CK levels ($P < 0.05$) and 24 hr CK levels ($P < 0.05$) were significantly higher with carbohydrate ingestion than carbohydrate-protein-antioxidant ingestion. Post-exercise LDH levels ($P < 0.05$) and 24 hr LDH levels ($P < 0.05$) were significantly higher with carbohydrate ingestion than carbohydrate-protein-antioxidant ingestion. Peak muscle soreness levels 24 hr post-exercise were significantly higher with carbohydrate ingestion than carbohydrate-protein-antioxidant ingestion ($P < 0.05$). These findings demonstrate that the carbohydrate-protein-antioxidant beverage significantly attenuated post-exercise muscle damage and subjective muscle soreness, but the additional protein content did not improve time to exhaustion significantly.

Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents post-exercise muscle damage (Saunders, Luden, and Herrick, 2007)	To compare the effects of an oral-carbohydrate-protein gel against a carbohydrate only gel on cycling endurance performance and post-exercise muscle damage, and to compare responses between genders.	13 trained cyclists: 8 males (23.8 ± 7.4 yr, 74.8 ± 10.1 kg, 61.5 ± 4.2 ml/kg/min), 5 females (24.8 ± 6.4 yr, 61.1 ± 7.0 kg, 51.3 ± 4.9 ml/kg/min)	Subjects performed two bouts of cycle ergometry to exhaustion at 75% $\text{VO}_{2\text{peak}}$. Subjects were randomly administered carbohydrate-protein or carbohydrate only gels every 15 minutes during exercise, and the same treatment with water immediately post-exercise. The protocol was repeated 7 – 14 days later with the alternate treatment beverage.	The time to exhaustion was significantly longer with ingestion of the carbohydrate-protein gel, than the carbohydrate-only gel ($P < 0.05$). There were no gender differences in time to exhaustion ($P = 0.98$). The increase in CK levels post-exercise was significantly lower with ingestion of the carbohydrate-protein gel ($P < 0.05$). These findings suggest that ingestion of a carbohydrate-protein gel during and after exercise improves cycling endurance performance, and attenuates muscle damage.
Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption (Valentine et al., 2008)	To examine the effects of carbohydrate-protein beverage ingestion on time to exhaustion and markers of muscle disruption compared to placebo and carbohydrate beverages matched for carbohydrate (CHO) and total calories	11 trained male cyclists (20.8 ± 2.4 yr, 75.2 ± 11.6 kg, 53.4 ± 7.2 ml/kg/min)	Subjects performed four separate cycle ergometry rides to exhaustion at 75% $\text{VO}_{2\text{peak}}$, at a cadence of 50 rpm. Subjects were randomly administered 250 ml of a placebo, a carbohydrate only (6%) beverage, a higher dosage (9.69%) of carbohydrate beverage, or a carbohydrate (7.75%) and protein (1.94%) beverage every 15 min until fatigue. Subsequent trials were performed 5-7 days apart with the alternate treatment beverages.	The cycling times to exhaustion were significantly longer with the carbohydrate-protein and the higher dosage of carbohydrate beverages, than the placebo ($P < 0.05$). There were no significant differences in times to exhaustion between the lower dosage carbohydrate beverage and the placebo, or between the carbohydrate-protein beverage, the higher dosage of carbohydrate beverage, and the lower dosage of carbohydrate beverage. Post-exercise plasma creatine kinase and serum myoglobin were lower during the carbohydrate-protein trial than all the other treatments. The number of 24hr post-exercise leg extensions performed at 70% of 1 RM was significantly greater in the carbohydrate protein trial than all other treatments. These findings suggest caloric differences may account for improved endurance performance with carbohydrate-protein beverages, whereas improvements in muscle recovery variables are independent of caloric content and carbohydrate.

Carbohydrate & carbohydrate + protein for cycling time-trial performance (Osterberg, Zachwieja, and Smith, 2008)	To determine if carbohydrate-protein ingestion significantly improves cycling time-trial performance relative to placebo and carbohydrate	13 trained male cyclists (31.2 + 2.4 yr, 73.4 + 9.0 kg, 56.0 + 6.9 ml/kg/min)	Subjects performed three separate experimental trials of constant-load cycle ergometry for 120 min, followed immediately by a time-trial in which they completed a set amount of work as quickly as possible (7 kJ/kg). Subjects were randomly administered 250 ml of a placebo, a carbohydrate only (6%) beverage, or a carbohydrate (7.5%) and protein (1.6%) beverage every 15 min during the constant-load ride. Subsequent trials were performed 5-7 days apart with the alternate treatment beverages.	The time trial performance was significantly faster ($P < 0.05$), and the power output during the time trial was significantly higher ($P < 0.05$) with ingestion of the carbohydrate-only beverage, compared to the placebo. The time trial performance and power output during the time trial were not significantly different with ingestion of the carbohydrate-protein beverage, than either the carbohydrate-only or placebo beverage. The RPE, heart rate, and percent hydration during the constant-load ride were not different between all three treatments. These findings suggest that the carbohydrate-protein beverage provide no additional benefit to cycle time-trial performance.
Carbohydrate and protein hydrolysate co-ingestion improves late-exercise time-trial performance (Saunders et al., 2009)	To compare the effects of a carbohydrate-casein hydrolysate beverage against a carbohydrate only beverage on cycling time trial performance, and markers of muscle disruption and recovery.	13 trained male cyclists (25.3 + 2.4 yr, 73.0 + 2.6 kg, 60.8 + 1.6 ml/kg/min)	Subjects performed two simulated 60 km cycle time trials with a 5% grade in the last 5 km. Subjects were administered 200 ml randomly assigned carbohydrate-only beverage (6%) or carbohydrate-casein hydrolysate beverage (6% + 1.8%) every 5 km during exercise, and 500 ml of the same treatment immediately post-exercise. The protocol was repeated 7 – 10 days later with the alternate treatment beverage.	The riding times in the final 20 km of the time trials and the last 5 km with 5% grade were significantly faster with ingestion of the carbohydrate-casein hydrolysate beverage, when compared to the carbohydrate-only beverage ($P < 0.05$). Post-exercise plasma CK levels and muscle soreness ratings were significantly elevated with ingestion of the carbohydrate-only beverage ($P < 0.05$), but not with carbohydrate-casein hydrolysate beverage. These findings suggest that the addition of casein hydrolysate to a carbohydrate beverage significantly improves late time-trial cycling performance and attenuates post-exercise elevations in plasma CK and muscle soreness.

<p>Influence of protein hydrolysate dosage on effects of carbohydrate and protein ingestion during exercise (Moore et al., 2009)</p>	<p>To compare the effects of carbohydrate beverages with varying amounts of protein hydrolysate content on endurance exercise and markers of muscle disruption.</p>	<p>10 trained male cyclists and runners: 25.7 ± 3.4 yr, 72.6 ± 3.2 kg, 51.7 ± 2.6 ml/kg/min (bike VO_2peak), 54.3 ± 2.3 ml/kg/min (treadmill VO_2peak)</p>	<p>Subjects performed three simulated duathlons, consisting of an 8 km treadmill run at 80% VO_2max, simulated 50 km cycle ergometer ride at 70% VO_2max, and subsequent treadmill run at 80% VO_2max until fatigue. Subjects were administered 1.4 L of randomly assigned carbohydrate-only beverage (6%), carbohydrate + 1% protein hydrolysate beverage, or carbohydrate + 2% protein hydrolysate during the cycle exercise. Subsequent trials were performed 6 – 10 days apart with the alternate treatment beverages.</p>	<p>The time to exhaustion was significantly longer in the subsequent treadmill run with ingestion of the carbohydrate + 1% protein hydrolysate beverage, than with the carbohydrate-only beverage ($P < 0.05$). Post-exercise plasma CK levels were significantly lower with ingestion of the carbohydrate + 2% protein hydrolysate beverage, than the other two treatments. Post-exercise muscle soreness ratings were significantly lower with ingestion of the carbohydrate + 2% protein hydrolysate, than with the carbohydrate-only beverage. The decline in pre- to post-exercise peak isometric force (MVC) was significantly greater with the carbohydrate-only ingestion, than the other two treatments. These findings suggest the addition of small amounts of protein hydrolysate to a carbohydrate beverage improves time to fatigue in endurance exercise, whereas a larger amount of protein hydrolysate attenuates muscle damage associated with heavy endurance exercise.</p>
<p>Added Protein Maintains Efficacy of a Low-Carbohydrate Sports Drink (Martinez-Lagunas et al., 2010)</p>	<p>To investigate the aerobic capacity characteristics of an isocaloric carbohydrate-protein beverage, and a low-calorie carbohydrate-protein beverage against a 6% carbohydrate sports beverage.</p>	<p>12 (5 females and 7 males) trained cyclists (28.3 ± 1.4 yr, 65.7 ± 2.3 kg, 57.3 ± 2.7 ml/kg/min),</p>	<p>Subjects performed four separate rides between 55 – 75% VO_2max for 2.5 hours, and then at 80% VO_2max until fatigue. Subjects were randomly administered 255.4 ± 9.1 ml of a placebo, a carbohydrate only (6%) beverage, a higher dosage of carbohydrate (4.5%) and protein (1.15%) beverage, and a lower dosage of carbohydrate (3%) and protein (0.75%) beverage every 20min. Subsequent trials were performed 7 days apart with alternate treatment beverages.</p>	<p>The times to exhaustion were significantly longer in the carbohydrate-only, high and low dosages of carbohydrate-protein beverages, than the placebo ($P < 0.05$), with no significant differences between the 3 treatments. These 3 treatments also reported elevated blood glucose, plasma insulin, carbohydrate oxidation, and lower levels of free fatty acids, rates of fat oxidation, RPE compared to the placebo. This study suggests adding protein to sports recovery drinks is effective in enhancing aerobic capacity, while limiting carbohydrate and caloric intake.</p>

Table 2.2: Carbohydrate and Protein and Post-Exercise Recovery.

Articles	Problem/Question studied	Participants	Procedures	Findings
Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage (Saunders, Kane, and Todd, 2004)	To compare the effects of a carbohydrate-protein beverage against a carbohydrate-only beverage on cycling endurance performance and post-exercise muscle damage.	15 trained male cyclists (20.9 ± 3.3 yr, 73.3 ± 6.4 kg, 52.6 ± 10.3 ml/kg/min)	Subjects performed two trials of cycle ergometry to exhaustion (at 75% and 85% VO_2 peak respectively, 12 – 15 hr apart). Subjects were administered 1.8 ml/kg bodyweight of randomly assigned carbohydrate or carbohydrate-protein beverage every 15 minute during exercise, and 10 ml/kg bodyweight of the same treatment post-exercise. The protocol was repeated 7 – 14 days later with the other treatment beverage.	The time to exhaustion was significantly longer in the subsequent performance ride with consumption of the carbohydrate-protein beverage, when compared to the carbohydrate-only beverage ($P < 0.05$). Peak post-exercise CK levels (12 – 15 hr after the initial ride) were significantly reduced with ingestion of the carbohydrate-protein beverage. These findings demonstrate significant improvements in subsequent cycling time to exhaustion and reductions in muscle damage in cyclists with the addition of protein calories in carbohydrate beverages.
Recovery from run training: efficacy of a carbohydrate-protein beverage? (Millard-Stafford et al., 2005)	To determine if the increased calories from protein content or additional carbohydrate content provide ergogenic benefits over traditional CHO sports drinks during recovery from heavy aerobic exercise.	8 (5 females and 3 males) trained endurance runners (28.6 ± 6.6 yr, 59.3 ± 8.1 kg, 56.5 ± 5.9 ml/kg/min)	Subjects performed a 21 km outdoor run at 70% VO_2 max, followed immediately by a run to fatigue at 90% VO_2 max on a treadmill. Subjects were then administered one of three treatments during a 2 hour recovery period: a carbohydrate (8%) and protein (2%) beverage, a isocaloric 10% carbohydrate beverage, or a commercially available 6% carbohydrate drink. Subjects then performed the treadmill run to fatigue after 2 hours, and were administered the same test beverage post-exercise. The subjects finally performed a 5 km time trial 24 hours later. Subsequent trials were performed at least 7 days apart with the alternate treatment beverages.	Blood glucose and insulin were significantly elevated during the 2 hour recovery period with ingestion of the isocaloric 10% carbohydrate beverage ($P < 0.05$). There were no significant improvements from the first to second run to fatigue, or the 24 hour 5 km time trial with all three treatment beverages. There were no significant differences in plasma CK levels in all three treatment beverages. Muscle soreness ratings were significantly lower with the carbohydrate-protein beverage, compared to the isocaloric 10% carbohydrate drink. These findings suggest that while additional calories from carbohydrate or protein above those provided in commercially available drinks do not improve subsequent endurance performance after recovery; the additional protein content may help to attenuate muscle soreness.

Chocolate milk as a post-exercise recovery aid (Karp, 2006)	To determine the efficacy of chocolate milk as a recovery beverage on subsequent endurance performance following glycogen-depleting exercise.	9 trained male cyclists (22.1 ± 2.0 yr, 73.0 ± 4.6 kg, 65.0 ± 9.0 ml/kg/min)	Subjects performed three separate glycogen depleting trials at 90, 80, 70% of their maximal power for 2 min intervals on the cycle ergometry, followed by a subsequent performance ride to exhaustion at 70% VO_2max after a 4 hr recovery. Subjects were randomly administered isovolumic amounts of low-fat chocolate milk, a fluid replacement drink, or a carbohydrate replacement drink immediately post-exercise and at 2 hr into the recovery period. Subsequent trials were performed at least 7 days apart with the alternate treatment beverages.	The times to exhaustion and total work performed in the subsequent ride were significantly greater with the ingestion of the low fat chocolate milk and the carbohydrate replacement drink, compared to the fluid replacement beverage ($P < 0.05$). There were no significant differences in heart rate and RPE in the subsequent ride, as well as in post-exercise blood lactate for the glycogen depleting exercise and subsequent ride among all three treatments. These findings suggest that chocolate milk is an effective recovery beverage for subsequent endurance exercise.
The influence of carbohydrate and protein ingestion during recovery from prolonged exercise on subsequent endurance performance (Betts et al., 2007)	To determine if the potential ergogenic benefit of combined carbohydrate-protein ingestion during recovery from heavy endurance exercise is due to the protein content itself or from the additional calories.	6 trained male endurance runners (21 ± 3 yr, 72.6 ± 8.4 kg, 61.4 ± 7.3 ml/kg/min)	Subjects performed three separate trials of 90 min treadmill run at 70% VO_2max . Subjects were then administered one of three treatments at 30 min intervals during a 4 hour recovery period: a carbohydrate and whey protein isolate beverage, a lower carbohydrate concentration beverage (0.8 g per kg of bodyweight), or a higher carbohydrate concentration beverage (1.1 g per kg body mass). Following recovery, subjects then performed a run to exhaustion at 70% VO_2max . Subsequent trials were performed at least 7 days apart with the alternate treatment beverages.	The mean run times to exhaustion following the 4 hour recovery were significantly longer with the ingestion of the carbohydrate-protein beverage, and the higher carbohydrate dosage drink ($P = 0.05$). The exercise capacity during the run time to exhaustion was significantly improved with ingestion of the carbohydrate-protein beverage, when compared with a matched quantity of carbohydrate alone ($P = 0.02$). There were no significant differences in time to exhaustion between the carbohydrate-protein beverage and the higher carbohydrate concentration beverage. These findings suggest that additional calories from protein or carbohydrate content in recovery beverages prolongs time to exhaustion and improves exercise capacity in subsequent endurance performance, regardless of the origin of the additional energy source (sucrose or whey protein isolate).

Increased carbohydrate oxidation after ingestion carbohydrate with added protein (Betts et al., 2008)	To examine the metabolic impact of post-exercise carbohydrate-protein supplement ingestion between two running trials performed in the same day.	6 endurance trained males (22 + 1 yr, 73.8 + 6.7 kg, 61 + 6 ml/kg/min)	Subjects performed two separate trials of 90 min treadmill run at 70% VO ₂ max. Subjects were then administered either a carbohydrate and whey protein isolate solution, or a carbohydrate-only solution at 30 min during a 4 hour recovery period. Following recovery, subjects then performed a 60 min treadmill run at 70% VO ₂ max. The entire protocol was repeated 14 days later with the other treatment beverage.	The serum insulin concentrations and median insulinemic responses were significantly higher with the carbohydrate-protein solution (P = 0.02, P = 0.04 respectively). Whole-body carbohydrate oxidation was significantly higher during the subsequent run with the carbohydrate-protein solution (P < 0.01). These findings suggest that the addition of protein content in a carbohydrate recovery beverage may increase the oxidation of extramuscular carbohydrate sources without altering the rate of muscle glycogen degradation during subsequent endurance performance.
Effects of an Amino Acid–Carbohydrate Drink on Exercise Performance After Consecutive-Day Exercise Bouts (Skillen et al., 2008)	To examine the two week effects of daily combined carbohydrate and amino acid supplementation on exercise performance, muscle damage, and muscle soreness from consecutive bouts of cycling endurance performance.	12 trained male cyclists and tri-athletes (28.5 + 2.1 yr; 73.8 + 4.0 kg and 60.8 + 1.9 ml/kg/min during carbohydrate-only treatment; 73.6 + 4.1 kg and 60.2 + 2.1 ml/kg/min during carbohydrate-amino-acid treatment)	Subjects performed a 90 min cycle ride at 70% VO ₂ peak, followed by a ride to exhaustion at 85% VO ₂ max. Subjects were then supplemented with a 3.6% carbohydrate and 1% amino acid beverage, or a 4.6% isocaloric carbohydrate-only beverage for 2 weeks. Subjects then repeated the above cycle rides for 2 consecutive days after the 2 weeks of supplementation. The entire protocol was repeated 14 days later with the other treatment beverage.	The time to exhaustion between the 1 st and 2 nd consecutive rides decreased significantly with the carbohydrate-only treatment, and increased significantly with the carbohydrate-amino acid treatment (P < 0.05). The decrease in vertical jump height on the 2 nd consecutive day was significantly attenuated with the carbohydrate-amino acid treatment (P < 0.05). Plasma CK levels, total fatigue score, and mood disturbance were significantly lower on the 2 nd consecutive day with the carbohydrate and amino acid beverage. These findings suggest that the addition of amino acids to a carbohydrate beverage after consecutive day exercise bouts may help maintain subsequent endurance performance, reduce muscle damage, and decrease fatigue.

Effect of dietary protein content during recovery from high-intensity cycling on subsequent performance and markers of stress, inflammation, and muscle damage in well-trained men (Rowland, 2008)	To examine the effects of a high carbohydrate, protein enriched post-exercise recovery diet on subsequent cycling endurance performance.	12 trained male cyclists (34 ± 10 yr, 75.9 ± 4.3 kg, 4.9 ± 0.6 L/min)	Subjects performed 3 high-intensity rides over 4 days: Day 1 comprised of 2.5 hour intervals, and days 2 & 4 comprised of repeat-sprint performances, with day 3 as a recovery day. Subjects were administered either the protein-enriched diet, or the control diet (equal servings of protein and fat) 4 hours post-exercise on days 1 & 2. The entire protocol was repeated 14 days later with the other treatment diet.	The mean sprint power was significantly higher on day 4 (4.1%, $P < 0.05$), with the protein-enriched diet. Overall plasma CK levels throughout the 4 days may experience a possible reduction with the protein-enriched diet, and were substantially lower on day 4 before exercise ($P = 0.08$). These findings suggest that while the addition of protein content in a post-recovery diet may not confer short-term (15 hour) benefits, it may improve delayed (60 hour) subsequent high-intensity sprint performance and attenuate delayed muscle damage.
Recovery from a cycling time trial is enhanced with carbohydrate-protein supplementation vs. Isoenergetic carbohydrate supplementation. (Berardi et al., 2008)	To examine the effects of ingesting a liquid carbohydrate-protein supplement during recovery from a cycling time trial on the subsequent 60 min ride on the same day, against an isoenergetic liquid carbohydrate supplement.	15 trained male cyclists: 7 receiving carbohydrate-protein supplementation (32.43 ± 8.85 yr, 74.76 ± 9.39 kg) and 8 receiving carbohydrate-only treatment (34.00 ± 9.74 yr, 78.98 ± 8.21 kg)	Subjects performed a cycle time trial for 60 min. The distance covered was recorded. Subjects were then administered 1 L of carbohydrate-protein or carbohydrate only liquid supplement at 10/60/120 min post-exercise. Following 6 hours of recovery, subjects repeated the 60 min cycle time trial.	The decrease in distance covered and decrease in power output during the subsequent time trial were significantly reduced in the carbohydrate-protein supplement group. The rate of fat oxidation was significantly higher in the carbohydrate-protein group ($P < 0.05$), despite a higher average workload. These findings suggest that ingestion of a liquid carbohydrate-protein supplement post-exercise increases fat oxidation, increases recovery and improves subsequent endurance performance in a cycling time trial.

Improved endurance capacity following chocolate milk consumption compared with 2 commercially available sport drinks (Thomas, Morris, and Stevenson, 2009)	To examine the effects of 3 isocaloric recovery beverages on subsequent endurance performance following glycogen depleting exercise	9 trained male cyclists (25.4 ± 8.0 yr, 72.8 ± 8.4 kg, 4.3 ± 0.4 L/min)	Subjects performed three separate glycogen depleting trials at 90, 80, 70, and 60% of their maximal power for 2 min intervals on the cycle ergometry, followed by a subsequent performance ride to fatigue at 70% maximal power after a 4 hr recovery. Subjects were randomly administered a 1 g/kg bodyweight of carbohydrate replacement drink, an isovolumic amount of fluid replacement drink, or an isocaloric amount of chocolate milk immediately post-exercise and at 2 hr into the recovery period. Subsequent trials were performed at least 7 days apart with the alternate treatment beverages.	The time to exhaustion was significantly longer with ingestion of the chocolate milk, than the carbohydrate replacement and fluid replacement beverages ($P < 0.05$). This finding suggests that when matched for caloric content with a carbohydrate replacement drink, chocolate milk is an effective recovery beverage for subsequent endurance exercise of moderate intensity.
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Table 2.3: Genotypes and Sport Performance.

Articles	Problem/Question studied	Participants	Procedures	Findings
Elite endurance athletes and the ACE I allele – the role of genes in athletic performance (Gayagay et al., 1998)	To identify genetic markers in the renin-angiotensin pathways that is associated with cardiovascular performance.	64 Australian national rowers (43 males, 21 females), and 118 controls	Blood samples were collected and DNA extractions were performed for genotyping analysis of the RAS polymorphisms. The ACE gene insertion (I) and deletion (D) polymorphism were detected by polymerase chain reaction conditions.	The rowers exhibited significantly higher frequency of the ACE I allele ($P > 0.02$), and significantly higher genotypic distributions of the homozygous ACE II genotype ($P = 0.03$) than the controls. There was a corresponding decreased frequency of the ACE D allele in the rowers. These findings suggest that a genetic factor associated with the ACE I allele may confer beneficial effects in human cardiovascular performances.
Human angiotensin I-converting enzyme gene and endurance performance (Myerson et al., 1999)	To examine the frequency distribution of the ACE I allele in elite distance runners and other elite athletes.	495 British elite athletes: 91 distance runners (48 males, 43 females), 404 athletes from 19 non-endurance dependent disciplines (219 males, 185 females), and 1906 controls.	Subjects' DNA were extracted from the buccal cells contained in 10 ml of saline mouthwash (0.9% sodium chloride solution). The ACE I and D polymorphisms were identified by polymerase chain reaction amplification of the polymorphic region.	There is a significant linear trend of increasing frequency ($P = 0.009$) in the ACE I allele with primarily anaerobic athletes (≤ 200 m), mixed aerobic and anaerobic athletes (400 – 3000 m), and primarily aerobic athletes (> 5000 m). The frequency of the ACE I allele was not significantly different among the non-endurance athletes from and the 19 disciplines, and the controls ($P = 0.526$). These findings show that the frequency of the ACE I allele is higher in among elite distance runners, and may increase with the distance run. This suggests that the ACE I allele is positively associated with elite endurance performance.

<p>ACTN3 genotype is associated with human elite athletic performance (Yang et al., 2003)</p>	<p>To examine the effects of the ACTN3 genotype on human athletic performance, and determine the influence of the ACTN3 genotype on variations in muscle function.</p>	<p>301 white elite athletes: 107 sprint athletes (72 males, 35 females), 194 endurance athletes (122 males, 72 females), and 436 controls (134 males, and 292 females).</p>	<p>Blood samples were collected and DNA extractions were performed for ACTN3 genotyping.</p>	<p>There were significant differences in allele frequencies between sprint athletes/endurance athletes and controls ($P < 0.010$). The allele frequency variations between sprint athletes and controls were significant for males ($P < 0.001$) and females ($P < 0.01$). Sprint athletes demonstrated a higher frequency of the RR genotype, a lower frequency of the RX genotype, and a lower frequency of the XX (ACTN3 null) genotype, while endurance athletes exhibited a slightly higher frequency of the XX genotype than controls. There were significant differences in allele frequencies between sprint and endurance athletes in both males ($P < 0.001$) and females ($P < 0.05$). This suggests that the R allele (ACTN3 positive) confers a beneficial effect on skeletal muscle function in generating forceful contractions at high velocities.</p>
<p>Mitochondrial DNA and ACTN3 genotypes in Finnish elite endurance and sprint athletes (Niemi and Majamäa, 2005)</p>	<p>To examine mtDNA haplogroup and subhaplogroup frequencies and differences in elite Finnish sprinters and endurance runners, and to assess the potential of ACTN3 genotypes as a confounding variable.</p>	<p>141 Finnish elite track and field athletes: 52 endurance athletes (800m – marathon, or walking), and 89 sprinters (100/400m or a field event).</p>	<p>Blood samples were collected and DNA extractions were performed for MtDNA haplogroup and subhaplogroup analyses, and ACTN3 genotyping.</p>	<p>There were significant frequency differences in mtDNA haplogroups between Finnish sprinters and endurance runners ($P = 0.039$). The frequencies of haplogroups J & K were higher in the sprinters, whereas the frequency of haplogroup I was higher in the endurance runners. The frequency of the ACTN3 577RR genotype was higher in the sprinters, whereas the frequency of the ACTN3 577XX genotype was higher in the endurance athletes. The 577XX genotype was inversely correlated to best sprint times in the sprinters ($P = 0.03$).</p>

<p>ACTN3 genotype in professional endurance cyclists (Lucia et al., 2006)</p>	<p>To examine the frequency distribution of ACTN3 in elite cyclists, and compare it against elite endurance runners, and sedentary controls.</p>	<p>52 elite male Caucasian cyclists (26.9 ± 0.4 yr, 178 ± 0.8 cm, 67.4 ± 0.9 kg), 50 elite male Caucasian endurance runners (26.8 ± 0.6 yr, 175.0 ± 0.9 cm, 61.6 ± 0.9 kg), and 123 sedentary Caucasian males (19 – 50 yr).</p>	<p>Gas-exchange measurements were obtained for the cyclists and runners, from maximal exercise tests to fatigue on the cycle ergometer and treadmill respectively, as indices of endurance performance. Blood samples were taken for DNA extraction and ACTN3 genotyping analysis (RR, RX, and XX).</p>	<p>There were no significant differences among elite cyclists, elite runners, and sedentary controls in all three ACTN3 genotype distributions ($P > 0.05$). There was a trend for a higher frequency of the ACTN3 XX genotype in elite cyclists. There were no significant differences between elite cyclists and elite runners of each genotype in the indices of endurance performance: VO_2max, ventilatory threshold (VT), and respiratory compensation threshold (RCT). This suggests that the ACTN3 polymorphisms do not confer advantages in endurance performances.</p>
<p>The effect of AMPD1 genotype on blood flow response to sprint exercise (Norman et al., 2008)</p>	<p>To determine if deficiency in skeletal muscle myoadenylate deaminase (mAMPD), due to variations in the AMPD1 gene, affect blood flow response to high intensity exercise.</p>	<p>15 subjects (21 – 32 yr)</p>	<p>Subjects perform a 30s Wingate test against a workload of 7.5% of body weight. Peak and average power over 5-s periods, mean power over entire 30-s duration, and power decline were recorded. Common femoral artery blood flow velocity and vessel diameter were measured pre- and 25 min post-exercise. Blood samples were collected 1/3/6/9/12/18/25 min following exercise.</p>	<p>Subjects homozygous and heterozygous for AMPD1 34C>T alleles MM/NM demonstrated significantly lower mean power than subjects homozygous for the AMPD1 normal allele NN (7.7 ± 0.6 W/kg vs 8.7 ± 0.2 W/kg, $P < 0.01$) as well as lower peak power, and larger power declines. MM/NM subjects demonstrated a significantly higher peak femoral arterial blood flow than NN subjects (5.44 ± 0.41 l/min vs 4.10 ± 0.31 l/min, $P < 0.001$), and a significantly faster normalization of arterial blood flow post-exercise (7.8 ± 1.1 min, vs 16.1 ± 1.4 min, $P < 0.001$). This was associated with a faster heart rate recovery in MM/NM subjects post-exercise. This suggests AMPD1 mutations contribute to diminished mAMPD, which would result in greater adenosine accumulation, thereby promoting exercise-induced hyperaemia in high-intensity exercise.</p>

<p>An ACTN3 knockout mouse provides mechanistic insights into the association between α-actinin-3 deficiency and human athletic performance (MacArthur et al., 2008)</p>	<p>To examine the mechanisms by which the ACTN3 R577X polymorphism influences human athletic performance through phenotypic analysis of the biochemical and physiological effects of ACTN3 deficiency in knockout mice.</p>	<p>Mice models (7-9 wk), 43 males (19 wild-type, 24 knockout – ACTN3 null allele), 83 females (39 wild-type, 44 knockout).</p>	<p>Grip strength was measured. Body composition was determined by DEXA. Transverse sections of the quadriceps, spinalis thoracis, extensor digitorum longus (EDL), and soleus muscles were analyzed for fiber type proportions and measured for size. Mitochondrial enzyme levels were measured to examine muscle metabolic activity. Fast-twitch EDL muscles were isolated to examine differences in contractile properties.</p>	<p>Average grip strength was significantly lower in knockout mice than wild-type mice ($P = 0.007$ males, $P = 0.02$ females). Total body weight was lower in male and female knockout mice than the wild-type mice. This was attributed to loss of lean mass rather than loss of fat mass. Fast glycolytic type IIb fibers responsible for ACTN3 in wild-type mice were significantly smaller in the quadriceps ($P < 0.05$), and spinalis ($P < 0.01$) of knockout mice. There were no significant differences in the total number of fibers in the EDL. This suggests that the reduction in muscle mass in knockout mice was attributed to reduction in specific fiber size rather than differences in fiber type distributions. The metabolic profile of knockout mice exhibited increases for enzymes in the glycolytic pathway, mitochondrial enzymes of the tricarboxylic acid cycle, electron transport chain enzyme, and mitochondrial enzymes involved in fatty acid oxidation. These changes in muscle metabolism suggest that in the absence of ACTN3, there is a shift from anaerobic towards aerobic pathways, as well as an increased reliance in β-oxidation of fatty acids. The twitch half-relaxation times in fast-twitch EDL muscles were significantly longer in knockout mice than wild-type mice ($P = 0.007$), and recovery from fatigue induction as determined by force generation capacity was greater in knockout mice muscle. These changes in contractile properties suggest a shift in the characteristics of fast fibers towards slow fibers with ACTN3 deficiency.</p>
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<p>ACTN3 R577X polymorphism and Israeli top-level athletes (Eynon et al., 2009)</p>	<p>To examine the frequency distribution of the ACTN3 R577X polymorphism between Israeli athletes and non-athletes, and among different sports (sprints and endurance events) at different levels of competition (top and national level) among the athletic Israeli population.</p>	<p>155 athletes (35.9 ± 12.2 yr), 119 males and 36 females, 74 distance runners (20 top level, 54 national level) and 81 sprinters (26 top level, 55 national level). 240 controls, 170 males and 70 females.</p>	<p>Genomic DNA was extracted from peripheral blood leukocytes for genotyping of the ACTN3 R577X polymorphism</p>	<p>The ACTN3 R577X genotype and allele frequencies were significantly different between sprinters and distance runners ($P = 0.00009$, and $P = 0.00007$ respectively), and between sprinters and controls ($P = 0.00003$, and $P = 0.00002$ respectively). The genotype frequencies were significantly different between the distance runners and controls, while the differences in allele frequencies between distance runners and controls demonstrated a trend towards significance ($P = 0.08$). Distance runners exhibited a significantly higher proportion of the XX genotype than sprinters ($P = 0.005$), and controls ($P = 0.006$). The R allele was more frequent in top level sprinters than national level sprinters. This suggests that ACTN3 R allele may be associated with top level sprint performance. There were no significant differences in genotype and allele frequencies in top level and national level endurance athletes ($P = 0.7$). This suggests that the ACTN3 X allele and XX genotypes may be additive rather than critical to endurance performance.</p>
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Table 2.4: Genotypes and Muscle Performance.

Articles	Problem/Question studied	Participants	Procedures	Findings
Angio-tensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload (Folland et al., 2000)	To examine the effect of the ACE-D allele on the response of human skeletal muscle to functional overload from strength training.	33 males (21.4 ± 0.5 yr, 75.9 ± 2.1 kg, 180 ± 1 cm) body mass index, 23.3 ± 0.6 kg/m ²)	Subjects participated in a 9-week strength training program of the quadriceps muscle, with each leg randomly assigned to isometric or dynamic strength training. Epithelial cells were collected through saline mouth rinses for genotyping analysis. Baseline and post-training isometric strength (MVC) of the quadriceps muscle was measured. Additional strength measurements include peak isokinetic torque at four angles and three velocities	Subjects with the ACE-D allele demonstrated significant improvements in quadriceps muscle strength (MVC) with isometric strength training ($P < 0.05$), and demonstrated a trend for greater increases in all isokinetic strength measurements. The interaction between training and genotype was non-significant with dynamic strength training. The interaction between genotype and training was consistent in all strength measurements for both types of training (ACE ID > ACE DD > ACE II). This suggests that the renin-angiotensin system may play a role in the response of skeletal muscle to functional overload via the ACE ID polymorphism.
The ACE gene and muscle performance (Williams et al., 2000)	To examine associations between ACE genotypes and changes in the mechanical efficiency of skeletal muscle following physical training.	58 Caucasian army recruits (19.4 ± 0.3 yr, 1.78 ± 0.01 m, 71.3 ± 1.2 kg)	Subjects participated in a primarily aerobic 11-week physical training program. Blood samples were taken for DNA extraction and genotyping analysis. Baseline and post-training steady state oxygen uptake (VO_2) and respiratory exchange ratio (RER) were measured on a cycle ergometer exercise to determine the efficiency of muscular contraction (delta efficiency).	Baseline delta efficiency was independent of ACE genotypes ($P = 0.59$). Subjects homozygous for ACE II genotype documented significantly higher post-training delta efficiency than baseline ($P < 0.01$), and significantly higher increases in delta efficiency than subjects homozygous for ACE DD genotype ($P < 0.025$). This suggests that the ACE II genotype may be associated with an improved mechanical efficiency in trained skeletal muscles due to possible increases in slow-twitch muscle fibers, which are more efficient in slow contractions.

<p>The I allele of the angiotensin-converting enzyme gene is associated with an increased percentage of slow-twitch type I fibers in human skeletal muscle (Zhang et al., 2003)</p>	<p>To determine the association between ACE genotypes and skeletal muscle fiber type distribution.</p>	<p>41 untrained subjects (31 males, 10 females, 24 ± 3 yr)</p>	<p>Blood samples were taken for DNA extraction and genotyping analysis. Skeletal muscle samples were taken from the left vastus lateralis, and fiber type distribution was classified into type I, IIa and IIb.</p>	<p>Subjects homozygous for ACE II genotype exhibited significantly higher percentages of type I fibers, and significantly lower percentages of type IIb fibers compared with subjects homozygous for ACE DD genotype. Across the genotype groups, there are significant linear trends ($P < 0.01$) for decreased distribution of type I fibers (ACE II > ACE ID > ACE DD), and increased distribution of type IIb fibers (ACE II < ACE ID < ACE DD). This demonstrated that the ACE-I allele is associated with higher ratios of type I muscle fibers.</p>
<p>ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women (Clarkson et al., 2005)</p>	<p>To determine the associations between ACTN3 and muscle size, isometric strength, and dynamic strength following a 12-week resistance training program.</p>	<p>602 subjects (18 – 40 yr), 247 males and 355 females, 469 Caucasians, 28 African-Americans, 25 Hispanics, 55 Asians</p>	<p>Subjects participated in a 12-week resistance training program of their non-dominant elbow flexor/extensor. Blood samples were taken for DNA extraction and genotyping analysis. Baseline and post-treatment maximal isometric (MVC) and dynamic (1-RM) strength, and muscle size (cross-section area) of the elbow flexor were measured.</p>	<p>No significance between ACTN3 genotype and muscle size/strength in male subjects. Female subjects homozygous for the mutant ACTN3 XX allele exhibited significantly larger gains in absolute and relative 1-RM ($P < 0.05$). ACTN3 genotype is associated with 2% of baseline MVC and 1-RM strength gain ($P = 0.01$). This suggests ACTN3 may contribute to genetic variations in muscle response and adaptation to resistance exercise.</p>

ACE ID genotype and the muscle strength and size response to unilateral resistance training (Pescatello et al., 2006)	To examine associations among ACE insertion (I) and deletion (D) polymorphisms and the response to a 12-week resistance training program.	631 subjects (24.2 \pm 0.2 yr), 264 (41.8%) males and 367 (58.2%) females, 79.5% Caucasians, 4.1% African-Americans, 4.6% Hispanics, 8.1% Asians, 3.3% other	Subject participated in a 12-week unilateral upper-arm resistance training program of their non-dominant arms (5 exercises: bicep preacher curl, biceps concentration curl, standing biceps curl, overhead triceps extension, and triceps kickback). Exercise intensity started at 65 – 75% of 1-RM (3 sets x 25 repetitions), and was increased at week 5 (75 – 82% 1-RM, 3 sets x 8 repetitions) and week 10 (83 – 90% 1RM, 3 sets x 6 reps). Blood samples were taken for DNA extraction and genotyping analysis. Baseline and post-training maximal isometric (MVC) and dynamic (1-RM) strength, and muscle size (cross-section area) of the elbow flexor were measured.	Absolute and relative MVC increases were greater for ACE II/ID genotypes than ACE DD genotype in non-dominant/trained arms ($P < 0.05$), as well as dominant /untrained arms ($P < 0.05$) post-training. Absolute and relative increases in 1-RM and cross-section area were independent of ACE genotype groups in non-dominant arms post-training ($P \geq 0.05$). Absolute and relative increases in 1-RM and cross-section area were greater for ACE DD/ID genotypes than ACE II homozygotes in dominant arms post-training ($P < 0.05$). There were no differences in baseline muscle strength and size among the ACE genotype groups. This suggests that the ACE ID genotype may be more associated with the contralateral education and learning effects of unilateral resistance training, than with muscular strength and hypertrophic adaptations.
ACTN3 (R577X) genotype is associated with fiber type distribution (Vincent et al., 2007)	To examine the associations between ACTN3 polymorphisms and skeletal muscle fiber type distribution, and fast-velocity knee extension strength.	90 males (21.7 \pm 2.3 yr, 73.3 \pm 8.6 kg)	Blood samples were taken for DNA extraction and genotyping analysis. Isometric knee extensor strength (45°) was measured at different dynamic velocities (100 – 300°/s). Skeletal muscle samples were taken from the right vastus lateralis, and fiber type distribution was classified into type I, IIa and IIx. Protein content in the different fiber types was determined with assays.	Subjects homozygous for the ACTN3-R allele recorded significantly higher relative dynamic quadriceps torques at 300°/s, compared with subjects homozygous for XX ($P < 0.05$). Subjects homozygous for RR exhibited significantly greater distribution of type IIx fibers than subject homozygous for XX ($P < 0.05$). α -actinin-3 protein content was higher in type IIx than in type IIa fibers. This suggests the differences in skeletal muscle fiber type characteristics between the two ACTN3 genotype groups are significant, and the association between the ACTN3 polymorphism and fiber type distribution may affect muscle power.

ACE genotype and the muscle hypertrophic and strength responses to strength training (Charbonneau et al., 2008)	To examine associations between ACE genotypes groups, muscle phenotypes, and strength responses to strength training in older adults.	243 subject (50 – 85 yr), 81 males, 139 females, 147 Caucasians, 81 African-Americans	Subjects participated in a 10-week unilateral knee extension training of their dominant leg. Blood samples were taken for DNA extraction and genotyping analysis. Baseline and post-training muscle strength of the knee extensor (1-RM) and quadriceps muscle volume (MV) were measured.	Baseline MV in subjects homozygous for ACE DD genotype was significantly greater in both trained ($P = 0.02$) and untrained legs ($P = 0.01$) than subjects homozygous for ACE II genotype. There were no significant differences in baseline 1-RM in both trained and untrained legs among the ACE genotype groups. Post-training 1-RM ($P < 0.01$) and MV ($P < 0.01$) improved significantly among all ACE genotype groups, and there were no genotype differences in post-training 1-RM and MV in both legs. This suggests that there is no association among the ACE genotype groups for muscle strength and muscle volume adaptations to strength training in older adults, and that the ACE genotype is more associated with baseline differences in muscle volume.
Angiotensin-converting enzyme genotype affects skeletal muscle strength in elite athletes (Costa et al., 2009)	To examine the association between ACE genotype groups and skeletal muscle baseline strength in elite male and female athletes.	58 elite athletes: 35 swimmers (19 males, 16 females, 18.8 ± 3.2 yr), 23 tri-athletes (15 males, 8 female, 18.7 ± 3.0 yr). The athletes were Olympic candidates and further classified either as short (≤ 200 m), or middle (400m – 1500m) distance athletes	Baseline blood samples were collected for DNA extraction and ACE genotyping. Baseline bilateral grip strength, and maximum height in squat jump and counter movement jump were recorded.	Male and female subjects homozygous for ACE DD alleles demonstrated significantly higher right grip strength ($P \leq 0.05$) compared to subjects homozygous to ACE II allele. Short distance subjects of both genders recorded higher scores in most strength measurements ($P < 0.05$) than middle distance subjects. Female subjects heterozygous for ACE ID allele recorded significantly higher right grip strength ($P = 0.032$) and counter movement jump ($P = 0.048$) than those homozygous for ACE II allele. This suggests that ACE D allele is associated with baseline muscle strength in elite athletes, especially elite female athletes.

Table 2.5: Genotypes and skeletal muscle damage/recovery.

Articles	Problem/Question studied	Participants	Procedures	Findings
The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy (Adams and Haddad, 1996)	To examine the time course between increases in IGF-1 peptide and mRNA expression, and skeletal muscle hypertrophy.	128 female Sprague-Dawley rats (205 ± 3 g).	60 rats were hypophysectomized to reduce circulating IGF-1 and GH. The normal and hypophysectomized rats were grouped into overload and control groups. Bilateral removal of the gastrocnemius and soleus muscles was performed to overload the plantaris muscles in the overload groups. At 3/7/14/28 days post-surgery, control and overloaded rats were killed with an overdose of pentobarbital sodium. Plant muscles from both legs were excised for subsequent analysis.	Peak muscle IGF-1 peptide levels in normal/hypophysectomized rats occurred after 3/7 days of overloading, and were demonstrated a 4.1/6.2-fold increase against controls. These increases in muscle IGF-1 preceded the following hypertrophic response: Plantaris mass, myofiber size, and protein to bodyweight ratio were significantly higher with overloading in normal and hypophysectomized rats than the control groups ($P < 0.05$). The total DNA content of the plantaris muscles increased significantly in normal and hypophysectomized rats with overloading. The relationship between IGF-1 peptide levels and total DNA content in the plantaris muscle was significant in normal ($r = 0.53$, $P = 0.0001$) and hypophysectomized ($r = 0.73$, $P > 0.0001$) rats with overloading. These findings suggest that rats with normal and severely attenuated levels of IGF-1 increase their expression of local IGF-1 as a response to functional overload, which may contribute to the muscle hypertrophy response, through mobilization of satellite cells to provide increases in muscle DNA.

Developmental expression and location of IGF-I and IGF-II mRNA and protein in skeletal muscle (Gerrard et al., 1998)	To examine the role of IGF in skeletal muscle development in vivo.	Twenty one 30/44/59/68/75/89/109 day-old porcine fetuses, three 21 day old neonatal pigs, and three 6 month-old adult pigs.	Muscle and tissue samples were obtained from the semi-tendinosus of a limb and liver respectively for mRNA analysis. Muscle samples were taken from the contralateral pelvic limb for in situ hybridization.	Peak levels of muscle IGF-II occurred in 59 day old porcine fetuses and declined thereafter ($P < 0.05$). Maximal levels of muscle IGF-I occurred around birth ($P < 0.05$). Expression of IGF-I and IGF-II mRNA was localized to developing muscle fibers, as there was little signal was found in the surrounding connective tissues. These findings suggest that IGF-I and -II are primarily expressed and produced in muscle cells within developing muscle tissue, and hence, support fetal muscle development.
IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle (Chakravarthy, Davis, and Booth, 2000)	To examine the effects of repeated cycles of atrophy and re-growth on the capacity of satellite cells to replicate in aged skeletal muscle.	30 pathogen-free Fischer 344/Brown Norway F1 hybrid (FBN) male rats (3/8/31 mo) were used to determine the effects of normal aging on satellite cells, and 36 pathogen-free FBN male rats (25 – 26 mo, 523 ± 10 g) were used to determine the effects of repeated bouts of atrophy and re-growth on muscle mass, satellite cells.	Muscle samples were obtained from the gastrocnemius in the hind limbs bilaterally to isolate and culture satellite cells. Three cycles of hind-limb immobilization with plaster casts and intervening recovery periods were administered to promote atrophy and re-growth in skeletal muscle.	There were significant losses in gastrocnemius muscle mass and in the proliferative potential of the resident satellite cells after one bout of immobilization ($P < 0.05$), both of which did not recover from their decreased levels after either the first 3-week or subsequent 9-week recovery period. The infusion of IGF-I onto the atrophied gastrocnemius muscle for two additional weeks after the 9-wk recovery period rescued 46% of the lost muscle mass and significantly increased proliferation potential of the satellite cells ($P < 0.05$). These findings demonstrate the beneficial effects of local infusion of IGF-I to activate satellite cells in atrophied muscles.

Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise (Hameed et al., 2003)	To examine the acute effects of resistance weightlifting exercise on the different IGF-I polymorphisms in human skeletal muscle.	8 young men (29.5 ± 1.5 yr, 81.1 ± 2.4 kg, 179.3 ± 1.8 cm), and 7 elderly men (74.4 ± 1.8 yr, 74.7 ± 2.1 kg, 177 ± 2.3 cm)	Subjects performed 10 sets of 6 repetitions of single-leg knee extensor exercise at 80 % of their 1-RM. Muscle biopsy samples were taken from the vastus lateralis of the test leg and control leg 2.5 hr post-exercise. Total RNA was extracted from both test and control muscle samples, and transcribed into cDNA. mRNA was coded for ACTN, IGF-IEa, IGF-IEc (MGF) and MyoD.	Resting levels of IGF-IEa mRNA were significantly higher than MGF mRNA. There were no differences in resting levels of MGF mRNA and IGF-IEa mRNA between the young and elderly subjects. The resting levels of MyoD mRNA were significantly higher in the elderly subjects. With resistance exercise, the levels of MGF mRNA were significantly higher in the exercised legs than control legs of the younger subjects, but not the elderly subjects. There were no significant differences in the levels of IGF-IEa mRNA and MyoD mRNA between the control and exercised legs in both subject groups with resistance exercise. The proportion of myosin heavy chain II (MHC-II) were significantly lower ($P < 0.05$) in the quadriceps of the elderly adults. These findings suggest that resistance training attenuate the MGF response in elderly adults, whereas MGF and IGF-IEa isoforms are regulated differentially in young skeletal muscle.
Mice deficient in plasminogen activator inhibitor-1 have improved skeletal muscle regeneration (Koh et al., 2005)	To examine the role of the plasminogen system in skeletal muscle regulation and regeneration, and specifically, to test if deficiency in plasminogen activator inhibitor-1 (PAI-1), a primary inhibitor of urokinase-type plasminogen activator (uPA), would improve skeletal muscle regeneration.	uPA and PAI-1 null mice	Mice were anesthetized and isometric force measurements were recorded in situ. 10 μ l of cardiotoxin was injected into three locations of the extensor digitorum longus muscle. Mice were allowed to recover, and isometric force measurements were recorded 1 – 20 days post injection.	PAI-1 null mice exhibited greater expression of MyoD and developmental protein, and more rapid recovery of muscle morphology, protein levels, and function post-treatment compared to wild-type mice. The accelerated rate of recovery in PAI-1 mice is associated with greater accumulation of macrophages post-treatment. This suggests inhibition of PAI-1 in mice attenuates plasminogen activator activity, and stimulates skeletal muscle regeneration.

<p>ACTN3 and MLCK genotype associations with exertional muscle damage (Clarkson et al., 2005)</p>	<p>To examine if variations in the genes coding for myofibrillar proteins involved in skeletal muscle function influence responses to exertional muscle damage through eccentric resistance exercise</p>	<p>157 subjects (age of 24.1 ± 5.2 yr, height of 170.8 ± 9.9 cm, body mass of 73.3 ± 17.0 kg), 78 males and 79 females, 115 Caucasians, 4 African-Americans, 6 Hispanics, 20 Asians, 11 “other”</p>	<p>Subjects performed 50 maximal eccentric elbow flexor contractions (2 sets x 25 repetitions, 5 min interval). Maximal isometric strength (MVC) was tested pre- and immediately post-exercise and 4/7/10 days post-exercise. Blood samples were taken pre- and 4/7/10 days post-exercise for CK and Mb analysis, as well as DNA extraction and genotyping</p>	<p>ACTN3 R577X is associated with baseline CK activity, and explained 3.5% of all variation within baseline CK activity; no association with change in strength/blood proteins post exercise. MLCK 49T (homozygous rare allele found in 3.9% of subjects); greater increases in CK ($28,934$ U/l) and Mb (937 ng/ml). MLCK C37885A (heterozygous rare allele found in 22.3% of subjects): 57% strength loss, greater increases in CK.</p>
<p>ACE ID genotype affects blood creatine kinase response to eccentric exercise (Yamin et al., 2007)</p>	<p>To determine the role of the skeletal muscle rennin-angiotensin system (RAS), specifically the ACE ID genotype, in the regulation of exertional muscle injury through eccentric resistance exercise.</p>	<p>70 physical education students (25 ± 3 yr, 171 ± 8 cm, 67 ± 10 kg), 42 males and 28 females</p>	<p>Subjects performed 1 set of 50 maximal eccentric elbow flexor contraction of their non-dominant arm. Blood samples were taken pre- and 3/24/48/72/96/120/168 hr post-exercise for CK activity and genotype analysis</p>	<p>The ACE ID genotype was the most powerful independent determinant of peak CK activity (95% confidence interval, $P = 0.02$). The homozygous ACE II allele was associated with the highest peak CK activity ($8,882 \pm 2,361$ U/l) and the largest increase in CK activity ($8,735 \pm 2,352$ U/l). Peak CK activity and increases in CK activity were intermediate in the heterozygous ACE ID allele ($4,454 \pm 1,105$ U/l, $4,296 \pm 1,108$ U/l), and lowest in the homozygous ACE DD allele ($2,937 \pm 753$ U/L, $2,778 \pm 757$ U/l). This suggests that the ACE II genotype may be associated with the development of exertional rhabdomyolysis, whereas the ACE DD genotype may protect against exercise-induced muscle injury.</p>

<p>IGF-II gene region polymorphisms related to exertional muscle damage (Devaney et al., 2007)</p>	<p>To identify IGF polymorphisms associated with exertional muscle damage through eccentric resistance exercise.</p>	<p>151 subjects - 73 males (25.3 ± 5.4 yr, 170.5 ± 7.9 cm, 81.9 ± 17.2 kg) and 78 females (22.8 ± 4.6 yr, 159.2 ± 6.8 cm, 64.5 ± 11.4 kg), 73% Caucasians, 3% African-Americans, 4% Hispanics, 13% Asians, 7% “other”</p>	<p>Subjects performed 50 maximal eccentric elbow flexor contractions (2 sets x 25 repetitions, 5 min interval). Maximal isometric strength (MVC) was tested pre- and immediately post-exercise as the criterion measure. Muscle soreness was assessed pre and 3/4/7/10 days post-exercise via a visual analog scale. Blood samples were taken pre- and 4/7/10 days post-exercise for CK and Mb analysis, as well as DNA extraction and genotyping</p>	<p>IGF-II G13790C (homozygous rare allele found in 18.6% of subjects): 58% strength loss immediately post-exercise, increased serum CK activity at 7 days post-exercise (8818 U/l). IGF-II G17200A (homozygous rare allele found in 11.0% of subjects): 60% strength loss immediately post-exercise, increased CK activity at 7 days post-exercise (12261 U/l). IGF2AS A1364C (homozygous wild type found in 36.2% of subjects): 58% strength loss, 50mm soreness. IGF2AS G11711T (homozygous wild type found in 27.8% of subjects): 56% strength loss, 56mm soreness, increase in blood CK (17,160 U/l) and Mb (659 ng/ml).</p>
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Chapter III

Methods

Subjects

Fourteen endurance-trained male cyclists (18-45 yrs) were recruited from James Madison University and the surrounding area. The inclusion criteria included a minimum of three days a week of cycling for the past 2 months, and a peak aerobic capacity ($\text{VO}_{2\text{peak}}$) greater than 45 ml/kg/min. All subjects received a written consent form that described the risks and benefits associated with the study. After a description of the protocols, subjects signed the written consent form. James Madison University Institutional Review Board approved the experiment design before data collection began.

Procedures

The study design consisted of a pre-testing/screening phase, familiarization trial, and three treatment trials.

Pre-testing Phase

All potential subjects that met the study criteria completed the following assessments.

Body Mass and Height

Subjects had their body weight measured to the nearest 0.1kg and height measured to the nearest 0.5cm.

$\text{VO}_{2\text{peak}}/\text{W}_{\text{max}}$

Subjects completed an exercise test to determine their peak oxygen uptake ($\text{VO}_{2\text{peak}}$) and associated power output (W_{max}). The test began with subjects riding

a cycle ergometer at a self-selected pace, described as a “comfortable but not easy pace for an hour long ride.” After subjects selected their initial workload, power output was increased by 25 W every 2 minutes until the subject voluntarily requested to stop the test due to fatigue. Peak oxygen uptake and power output were measured during the test to determine if subjects met entry criteria for the study, and to establish intensities for subsequent exercise protocols.

Familiarization Trial

All subjects that met the inclusion criteria performed a familiarization trial within two weeks of the pre-testing assessment. The purpose of the familiarization trial was to determine if the exercise intensities were appropriate for each subject. The familiarization trial also allowed subjects to become acclimated to the protocol and establish the appropriate pace for the time-trial segment. Subjects were tested prior to the treatment phase in order to alter workloads and to ensure they can complete the treatment trial. Another purpose of the familiarization trial was to minimize any effects of learning and the repeated-bout effect, in order to reduce error variance between treatment trials. All subjects received the CHO recovery beverage during the familiarization trial (described below). The treatment phases began 7 to 10 days following the familiarization trial. No blood draws or muscle recovery measures were taken during the familiarization trial.

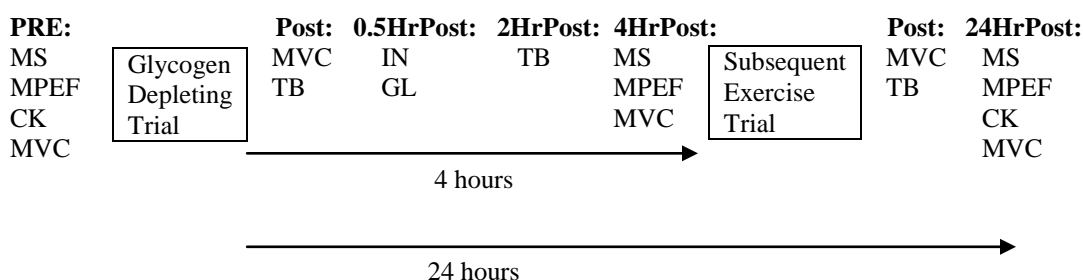
Treatment Phases

Treatment phases were similar to the familiarization trial with the addition of the supplementation treatment provided during the trial, and the inclusion of blood draws and

muscle recovery measures. The treatment phase consisted of a glycogen-depleting exercise, a recovery period and a subsequent time trial.

A visual timeline of events during each of the treatment phase is provided in **Figure 3.1** below.

Figure 3.1: Timeline for exercise trials and measurements of recovery.



MS=Muscle Soreness, MPEF= Mental/Physical Energy/Fatigue, CK=Creatine Kinase, MVC= Peak Isometric Torque, TB=Treatment Beverage, IN=Insulin, GL=Glucose

Glycogen-depleting exercise

Subjects performed a steady state warm-up for 10 minute at 60% W_{max} . After the initial 10 minutes of exercise, subjects completed a series of 2 minute high-intensity intervals. The high-intensity intervals consisted of 2 minutes at 95% W_{max} at a cadence of 70 rpm, followed by 2 minutes at 50% W_{max} . Once the cadence dropped below 70 rpm, the workloads for the high-intensity intervals were decreased to 85% W_{max} , with the cadence remaining at 70 rpm. Once the cadence dropped below 70 rpm again, the workload for the high-intensity intervals was further decreased to 75% W_{max} . The test was terminated when subjects failed to maintain a cadence of 70 rpm at this intensity. This protocol was designed to induce muscle glycogen depletion and moderate fatigue (Kuipers et al., 1987). Based on similar findings from Karp et al (2006) and Van Loon et al

(2000), the duration of the test was expected to last approximately 50-75 minutes, with 30-45 minutes of high-intensity intervals. All subjects were permitted to consume water *ad libitum* throughout the trial.

Recovery Period

Following the glycogen-depleting exercise there was a 4 hour period of recovery. Subjects consumed 750 ml of a recovery beverage immediately following this trial and again 2-hours post-exercise (see ‘treatment beverage’ below for description).

Subsequent Exercise Performance

After a 4 hour recovery period, subjects performed an endurance performance trial on a computerized cycle ergometer (VeloTron, Racermate, Inc). The start of the trial consisted of 20 minutes at a steady-state pace, with intensity corresponding to 60% W_{max} . The purpose of the steady state segment was to assess steady-state substrate utilization, and to ensure depletion of muscle glycogen. Following 20 minutes of steady-state exercise, subjects completed a simulated 20 km time trial. The last 5 km of the course consisted an uphill segment at 5% elevation. This was included in the protocol to allow an assessment of late-exercise performance.

All trials were performed at ambient room temperature (70-72 °F). The subjects were asked to void their bladders prior to all trials. A Windmere fan, set on ‘medium’ speed, was placed 2 meters from the handlebars of the ergometer for cooling purposes during the trial. Water was provided *ad libitum* throughout all

trials. Each subject was asked to provide maximal effort and treat each time trial as a competitive event. For consistency, no verbal encouragement was provided to the subjects during the time trial. The only feedback subjects received during the trials regarding performance were distance remaining and distance completed.

Subjects were given at least one week between trials to ensure complete recovery.

Participants were allotted 14 days to complete a trial in the event of unrelated illness or unforeseen circumstances, which occurred in four subjects.

Dependent measurements

Subsequent Exercise Performance

Exercise performance was measured using cycling time to completion for the 20 km time trial. Time was also recorded during the final 5km of the course.

Physiological Responses during Steady-State Exercise

Metabolic Measurements

Metabolic measurements were obtained during the glycogen-depleting trial and during the steady-state segment of the subsequent performance trial using a SensorMedics Spectra metabolic cart. These measurements were obtained after 3 minutes of cycling during the glycogen depleting trial (at 60% W_{max}) and after 3 minutes of cycling during the subsequent performance trial (also at 60% W_{max}). Expired respiratory gases were collected for 5 minutes at each time point. Average values were calculated using the final 3 minutes of each time period, following 2 minutes of breathing equilibration. Measurements obtained from the

expired gases included oxygen uptake (VO_2), ventilation (VE), and respiratory exchange ratio (RER).

Blood Glucose and Lactic Acid

Blood samples were obtained immediately prior to exercise, during the glycogen-depleting trial (at minute 8) and exercise performance trial (at minute 8), and 30 minutes post-exercise. Approximately 0.5 ml of blood was collected during each point via a finger-stick. An automated analyzer (YSI 2300 STAT glucose/lactate analyzer) determined glucose and lactate levels.

Serum Insulin

Thirty minutes after consuming the recovery beverage, 5 ml of blood was collected using a venous blood draw from an antecubital vein. The blood was collected in SST clot agent Vacutainer[®] tubes, centrifuged, and serum stored at -80 °C. Insulin levels were assessed from the serum through a commercially-available ELISA assay (ALPCO Diagnostics, Salam, NH).

Heart Rate

Heart rate was recorded using a Polar heart rate monitor (Lake Success, NY). Measurements were obtained at minute 8 of the glycogen-depleting trial and the subsequent exercise trial. Average heart was also recorded for the 20 km time trial and the final 5 km segment.

Ratings of Perceived Exertion (RPE)

Ratings of perceived exertion were obtained by having the subjects point to a corresponding level of exertion on the Borg RPE scale (6-20). The RPE recording was collected at the 8 minute mark during the glycogen-depleting and subsequent exercise. The following statement was read to the subjects to explain the RPE scale: *“Please describe your current level of exertion using the following scale. This level should represent your overall perception of effort, and not localized to a specific group of muscles, etc. For reference, a 6 would represent your effort when you are resting or watching TV, while 20 would represent the highest level of exertion you are capable of producing during exercise”*.

Muscle Recovery Variables

Muscle recovery variables included ratings of muscle soreness, mental and physical energy/fatigue ratings, isometric peak torque (MVC), and serum creatine kinase (CK). Muscle soreness ratings and mental/physical energy/fatigue ratings were collected during the following three time points; prior to the glycogen depletion trial (PRE), prior to the subsequent exercise performance trial (4HrPost) and 24 hours following the glycogen depletion trial (24HrPost). Serum CK was obtained twice: PRE and 24HrPost. Peak MVC was assessed five times: PRE, 4HrPost, 24HrPost, as well as immediately after the glycogen depleting trial and subsequent trial. A visual timeline of the muscle recovery measurements is provided in **Figure 3.1** above.

Serum Creatine Kinase (CK)

Serum CK was measured as an indirect indicator of muscle damage. Approximately 5ml of blood were obtained using a venous blood draw from an antecubital vein prior to exercise and 24-hr post exercise. Using a centrifuge, whole blood was spun at 7000 rpm to separate serum and stored in a -80 ° C freezer. Serum CK was analyzed using a Johnson and Johnson Vitro DT6011. The measurement device was calibrated prior to analyses using a reconstituted lyophilized calibration standard purchased from the manufacturer.

Muscle Function Test/Isometric peak torque (MVC)

Peak torque of the knee extensors were assessed to determine treatment differences in muscle function during recovery from exercise. Subjects were seated in a modified leg extension chair and asked to push as hard as possible against a shin pad that was connected to a force transducer. Each subject performed 3 to 6, 5-second repetitions at each time-point. There was a 1 minute rest between each repetition. MVC was also assessed immediately following both exercise sessions.

Mental and Physical Energy/Fatigue Ratings

The Mental and Physical State and Trait Energy and Fatigue Scales (MPSTEFS, O'Conner, 2004) were used to record ratings of mental and physical energy. Separate ratings of physical energy, physical fatigue, mental energy and mental fatigue were obtained using visual analog scales, with potential scores ranging from 0 to 300 for each rating.

Muscle Soreness Ratings

During the familiarization trial subjects received instruction on how to utilize the muscle soreness scale. The following statement was read to the subjects; “please describe your overall level of muscle soreness that you feel currently while performing normal daily activities (i.e. walking up or down stairs)”. The soreness rating used a 100mm visual analog scale; 0 indicated no muscle soreness and 100 indicated impaired movement due to muscle soreness.

Genotyping

Single nucleotide polymorphisms (SNPs) were evaluated in this study to determine if specific variations in these polymorphisms were associated with variations in muscle recovery variables, and examine their influence on individual responses to recovery beverages with protein. The following SNPs were evaluated in this study: ACTN3 R577X, IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, and IGF2AS G11711T. Approximately 4 ml of blood were obtained using a venous blood draw from an antecubital vein prior to the glycogen depleting exercise during the first trial, and mixed through inversion. DNA was isolated from each blood sample using the QIAamp® DNA Blood Mini Kit (Qiagen, CA). Subject identification information was removed for all DNA samples, which were subsequently shipped to the Research Center for Genetic Medicine at the Children’s National Medical Center in Washington D.C. for genotyping.

The following genotype polymorphisms were selected for genotyping based on their susceptibility to impaired muscle recovery as demonstrated by increased serum CK levels, strength loss, and muscle soreness following eccentric resistance exercises in prior research:

- 1) IGF-II C13790G (homozygous rare allele found in 18.6% of subjects): 58% strength loss immediately post-exercise, increased serum CK activity at 7 days post-exercise (8818 U/l) (Devaney et al., 2007).
- 2) IGF-II G17200A (homozygous rare allele found in 11.0% of subjects): 60% strength loss immediately post-exercise, increased CK activity at 7 days post-exercise (12261 U/l) (Devaney et al., 2007).
- 3) IGF2AS A1364C (homozygous wild type found in 36.2% of subjects): 58% strength loss, 50mm soreness (Devaney et al., 2007).
- 4) IGF2AS G11711T (homozygous wild type found in 27.8% of subjects): 56% strength loss, 56mm soreness, increase in blood CK (17,160 U/l) and Mb (659 ng/ml) (Devaney et al., 2007).

In addition, ACTN3 R577X was selected for genotyping due to its potential for demonstrating improved endurance performance in homozygous rare allele subjects (Yang et al., 2003; Niemi and Majamäa, 2005; Lucia et al., 2006; and Eynon et al., 2009).

Genotyping was performed using Applied Biosystem's Taqman allele discrimination assay using standard thermal cycling conditions, with genotypes called by the 7900HT Real-Time PCR System (Applied Biosystems, CA). For all the SNPs, the Applied Biosystem assay ID is located in **Table 3.1** or if the SNP was not available on the ABI website, a custom primer set was designed.

Table 3.1: TaqMan primer sets for SNPs tested.

Gene	SNP/ rs number	Forward Primer or ABI Assay #	Reverse Primer	WT allele probe (5' VIC)	MT allele probe (5' FAM)
IGF-II	C13790G rs3213221	C__31456535_10			
IGF2AS	G11711T rs7924316	C_29192859_10			
IGF-II	G17200A rs680	TGGACTTGAGT CCCTGAACCA	GTGCCCACCT GTGATTCTG	AGAGAAAA GAAGGGCC	AAAGAGAAA AGAAGGACC
IGF2AS	A1364C rs4244808	C__2981208_10			
ACTN3	R577X rs1815739	C____590093_1_			

Study Treatments

Recovery beverages were consumed immediately following the glycogen depleting exercise, 2 hours post glycogen depleting exercise, and immediately following the subsequent exercise performance trial. The study protocols were performed by each subject on three separate occasions (separated by 7-14 days each). Subjects consumed a different beverage for each trial. The beverages were provided in a randomly-counterbalanced, double-blinded design. Beverages were refrigerated at 5 °C from 2 hours prior to each trial until immediately prior to being consumed. Subjects were informed that the beverages consisted of the same amount of fluid with different nutrient composition, but were not informed the specific composition of the different beverages.

As this genotype study was conducted as part of a larger recovery beverage study, it adopted identical protocols that involved the administration of three different treatments. However, for the purposes of the current genotype study, only data collected from the carbohydrate-only treatment (CHO) and the high-carbohydrate low-protein treatment (HCLP) were analyzed.

Carbohydrate-only Beverage (CHO)

The CHO beverage contained 300 kcal, and 75g of carbohydrate, per 625 ml serving (12% by volume). To provide an isocaloric and isovolumetric (750 ml) comparison to the other beverage, the CHO beverage was consumed along with 125 ml of water.

High-Carbohydrate, Low-Protein Beverage (HCLP)

Each 250 ml serving of the HCLP beverage contained 300 kcal, 50g carbohydrate, 25g protein, and 0.5g of fat. To provide an isocaloric and isovolumetric (750 ml) comparison to the other beverage, the HCLP mixture was consumed along with 500 ml of water.

Dietary and Exercise Controls

Subjects were asked not to perform any heavy exercise 48 hours prior to each trial. A 48-hour diet and exercise log was completed by each subject preceding each treatment trial, and participants were instructed to maintain a consistent dietary and exercise habit between trials. No less than 12 hours prior to the start of the exercise, subjects consumed their final self-selected meal (dinner the evening prior to testing). After this meal,

subjects followed the standardized dietary protocol consisting of the following procedures:

- 1) No food or beverage intake between dinner and bedtime on the evening prior to the trial (water was consumed *ad libitum*).
- 2) On the morning of the exercise trials, subjects consumed a standardized breakfast two hours prior to the glycogen-depleting exercise trial. The meal was provided by the researchers, and consisted of 495-510 kcal, including 90-98g carbohydrate, 8-12g protein and 4.5-7.5g fat. In order to account for personal tastes, subjects chose from one of 4 meal choices, but the identical meal chosen was repeated across the four trials for each subject. These dietary restrictions ensured that no differences in nutrient intake occurred between the intervention periods, other than the treatments provided by the researchers.
- 3) No nutrients were consumed between the glycogen depleting trial and the subsequent exercise performance trial, other than the recovery beverages provided by the researchers.
- 4) No nutrients were consumed for 2 hours following the subsequent exercise performance trial, other than the recovery beverages provided by the researchers.
- 5) Subjects resumed their normal diets from 2 hours following the subsequent exercise performance trial through the 24HrPost measurement period. However, subjects recorded their dietary intake during this period, and were instructed to maintain similar dietary habits between the different trials.

Statistical Analyses

SPSS Version 17.0 was used to conduct statistical analyses. One-way Analysis of Variance (ANOVA) was used to analyze main effects for allele groups for time to completion for the 20 km and final 5 km during subsequent exercise performance. This

analysis was repeated for each of the five selected genotypes. Pairwise comparisons were performed to analyze specific differences in performance variables between allele groups for each genotype. To analyze genotype effects on markers of muscle recovery (serum CK levels, peak MVC, mental and physical energy/fatigue and muscle soreness ratings), repeated measures ANOVAs (RMANOVA) were conducted with 'time' as the within-subject factor (pre, post) and 'allele group' as the between-group factor. Post-hoc analyses were conducted using pairwise comparisons to analyze specific differences in change scores for each recovery variable between allele groups for each genotype. For specific allele groups identified as susceptible to impaired muscle recovery, RMANOVAs were conducted within the genotype group, with treatment (CHO, HCLP) and time (pre, post) as within-subject factors.

Chapter IV

Manuscript

INFLUENCE OF GENOTYPE VARIATIONS ON MARKERS OF SKELETAL
MUSCLE RECOVERY FROM HEAVY ENDURANCE EXERCISE, AND ON
RESPONSES TO CARBOHYDRATE-PROTEIN SUPPLEMENTATION

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Abstract

Purpose: This study examined the influence of genotype polymorphisms (specifically ACTN3 R577X, IGF-II C13790C, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T) on markers of skeletal muscle recovery following an acute bout of heavy endurance exercise, and assessed their role in determining individual responses to carbohydrate-protein supplementation. **Methods:** Twelve trained male cyclists completed repeated cycling trials, each consisting of an initial session of glycogen-depleting exercise, followed four hours later by a >1 hr time-trial on a computerized cycle ergometer (VeloTron, Racermate, Inc). Subjects were randomly administered a high-carbohydrate low-protein (HCLP), or carbohydrate-only (CHO) beverage, which was consumed immediately and 2-hours post glycogen depleting exercise, and immediately following subsequent exercise. Beverages were isocaloric and isovolumetric. Blood samples were obtained before the glycogen-depleting exercise of the first trial for genotyping analysis. Serum creatine kinase (CK), peak muscle function (MVC), mental and physical energy and fatigue ratings, and muscle soreness ratings were obtained as markers of muscle recovery at specific time points. Subjects completed these methods for both treatments in a double-blind protocol. **Results:** Changes in MVC were significantly different ($P < 0.05$) from baseline to 4-hours post glycogen depleting exercise among genotypes for the ACTN3 R577X, IGF-II C13790C, and IGF2AS A1364C single nucleotide polymorphisms (SNPs) during the CHO control trial. Changes in mental/physical energy/fatigue and muscle soreness ratings were significantly different ($P < 0.05$) from baseline to 24-hours post glycogen depleting exercise among genotypes for the ACTN3 R577X and IGF2AS G11711C SNPs during the CHO control trial. These

variables tended to improve with the HCLP treatment when subjects identified for the above genotype variations were analyzed, although the improvements were not statistically significant. Changes in serum CK were not significantly different ($P > 0.05$) from baseline to 4-hours post glycogen depleting exercise among genotypes analyzed.

Conclusions: Genotype variations led to significant differences in MVC, physical/mental energy/fatigue and muscle soreness ratings, which tended to improve with the HCLP treatment. There were no genotype effects for serum CK.

Key Words: Genotype, Carbohydrate, Protein, Muscle Damage, Recovery

Introduction

Prolonged high-intensity endurance exercise is associated with depleted glycogen stores and impairments in markers of muscle recovery. Traditionally, sports beverages containing water, carbohydrate, and electrolytes have been utilized by endurance athletes to enhance glycogen replenishment, facilitate muscle recovery, and improve aerobic performance (Hargreaves et al., 1984; Coyle and Coggan, 1984; Coggan and Coyle 1991; Coyle, 1992; 1992; Halson, 2004). Recently, the inclusion of protein into carbohydrate-based sport beverages to confer additional performance benefits has garnered considerable attention in the field of exercise science. The inclusion of protein content is proposed to enhance the rate of muscle recovery, as well as improve subsequent endurance performance (Saunders et al., 2004; Betts et al., 2007; Berardi et al., 2008; Rowland et al., 2008; and Skillen et al., 2008).

Current studies examining the efficacy of carbohydrate-protein supplementations have demonstrated mixed results. Some studies have documented improved performance in subsequent exercise bouts (Saunders et al., 2004; Betts et al., 2007; Berardi et al., 2008; Rowland et al., 2008; and Skillen et al., 2008), as well as reductions in markers of muscle damage and soreness (Saunders et al., 2004; Millard-Stafford et al., 2005; Romano-Ely et al., 2006; Rowland et al., 2008; and Skillen et al., 2008) with carbohydrate-protein ingestion. However, other studies have reported no differences in subsequent exercise performance between carbohydrate and carbohydrate-protein beverages (Millard-Stafford et al., 2005; Romano-Ely et al., 2006; Betts et al., 2007). In addition to the variations between studies, there appears to be considerable variations among individual subjects with respect to the potential efficacy of carbohydrate-protein

ingestion. For example, Combest et al. (2005) reported that 10 of 14 cyclists performing exhaustive exercise observed sizable attenuations in post-exercise serum CK levels ($\Delta\text{CK} = 975 \pm 590 \text{ U/L}$) with carbohydrate-protein ingestion, while the remaining four subjects were non-responders ($\Delta\text{CK} = -10.0 \pm 31.6 \text{ U/L}$) to carbohydrate-protein intake. Furthermore, the carbohydrate-protein ‘responders’ experienced improvements in subsequent exercise performance that were significantly greater than those who were ‘non-responders’.

The variations in findings within current literature suggest that some individuals are better responders to carbohydrate-protein supplementation than others, although the mechanism for the variations remains unclear. One possible explanation may lie in the role of specific genetic polymorphisms. Certain genotypes may predispose individuals to greater muscle damage following exercise. Hence, such individuals would potentially respond better to sports recovery beverages containing protein.

Current studies examining genotype associations with muscle damage have identified specific genotypes associated with increased skeletal muscle damage following acute exercise: MLCK C37885A (Clarkson et al., 2005), IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T (Devaney et al., 2007), and ACE ID (Yamin et al., 2007). However, these studies have primarily incorporated eccentric resistance exercise protocols. The association between genotypes and aerobic exercise-induced muscle damage is therefore not well understood. Hence, the present study seeks to determine if genetic polymorphisms (specifically IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, IGF2AS G11711T, and ACTN3 R577X) influence muscle damage/recovery variables following heavy endurance exercise. Differences in

susceptibility to cycling induced muscle damage could account for the varied responses to recovery beverage supplementation. The findings may help to determine the likelihood of individuals responding to recovery beverages containing protein. We hypothesize that 1) variations in genotypes among subjects will lead to significant differences in serum CK levels, peak muscle function, perceived physical/mental energy/fatigue, and perceived muscle soreness after acute intense aerobic exercise, and 2) subjects with genotypes associated with greater increases in the above muscle damage variables will derive greater reductions in these variables, and significant improvements in time to completion during a subsequent endurance performance exercise with the ingestion of high carbohydrate – low protein beverages.

Methods

Subjects

Fourteen endurance-trained male cyclists (18-45 yrs) were recruited from James Madison University and the surrounding area. The inclusion criteria included a minimum of three days a week of cycling for the past 2 months, and a peak aerobic capacity ($\text{VO}_{2\text{peak}}$) greater than 45 ml/kg/min. All subjects received a written consent form that described the risks and benefits associated with the study. After a description of the protocols, subjects signed the written consent form. James Madison University Institutional Review Board approved the experiment design before data collection began.

Procedures

The study design consisted of a pre-testing/screening phase, familiarization trial, and three treatment trials.

Pre-testing Phase

All potential subjects that met the study criteria completed the following assessments.

Body Mass and Height

Subjects had their body weight measured to the nearest 0.1kg and height measured to the nearest 0.5cm.

$\text{VO}_{2\text{peak}}/W_{\text{max}}$

Subjects completed an exercise test to determine their peak oxygen uptake ($\text{VO}_{2\text{peak}}$) and associated power output (W_{max}). The test began with subjects riding a cycle ergometer at a self-selected pace, described as a “comfortable but not easy pace for an hour long ride.” After subjects selected their initial workload, power

output was increased by 25 W every 2 minutes until the subject voluntarily requested to stop the test due to fatigue. Peak oxygen uptake and power output were measured during the test to determine if subjects met entry criteria for the study, and to establish intensities for subsequent exercise protocols.

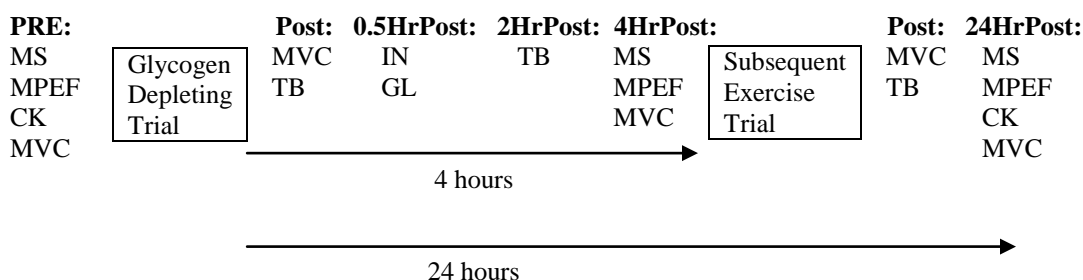
Familiarization Trial

All subjects that met the inclusion criteria performed a familiarization trial within two weeks of the pre-testing assessment. The purpose of the familiarization trial was to determine if the exercise intensities were appropriate for each subject. The familiarization trial also allowed subjects to become acclimated to the protocol and establish the appropriate pace for the time-trial segment. Subjects were tested prior to the treatment phase in order to alter workloads and to ensure they can complete the treatment trial. Another purpose of the familiarization trial was to minimize any effects of learning and the repeated-bout effect, in order to reduce error variance between treatment trials. All subjects received the CHO recovery beverage during the familiarization trial (described below). The treatment phases began 7 to 10 days following the familiarization trial. No blood draws or muscle recovery measures were taken during the familiarization trial.

Treatment Phases

Treatment phases were similar to the familiarization trial with the addition of the supplementation treatment provided during the trial, and the inclusion of blood draws and muscle recovery measures. The treatment phase consisted of a glycogen-depleting exercise, a recovery period and a subsequent time trial.

A visual timeline of events during each of the treatment phase is provided in **Figure 4.1** below.

Figure 4.1: Timeline for exercise trials and measurements of recovery.

MS=Muscle Soreness, MPEF= Mental/Physical Energy/Fatigue, CK=Creatine Kinase, MVC= Peak Isometric Torque, TB=Treatment Beverage, IN=Insulin, GL=Glucose

Glycogen-depleting exercise

Subjects performed a steady state warm-up for 10 minute at 60% W_{max} . After the initial 10 minutes of exercise, subjects completed a series of 2 minute high-intensity intervals. The high-intensity intervals consisted of 2 minutes at 95% W_{max} at a cadence of 70 rpm, followed by 2 minutes at 50% W_{max} . Once the cadence dropped below 70 rpm, the workloads for the high-intensity intervals were decreased to 85% W_{max} , with the cadence remaining at 70 rpm. Once the cadence dropped below 70 rpm again, the workload for the high-intensity intervals was further decreased to 75% W_{max} . The test was terminated when subjects failed to maintain a cadence of 70 rpm at this intensity. This protocol was designed to induce muscle glycogen depletion and moderate fatigue (Kuipers et al., 1987). Based on similar findings from Karp et al (2006) and Van Loon et al (2000), the duration of the test was expected to last approximately 50-75 minutes, with 30-45 minutes of high-intensity intervals. All subjects were permitted to consume water *ad libitum* throughout the trial.

Recovery Period

Following the glycogen-depleting exercise there was a 4 hour period of recovery. Subjects consumed 750 ml of a recovery beverage immediately following this trial and again 2-hours post-exercise (see ‘treatment beverage’ below for description).

Subsequent Exercise Performance

After the 4 hour recovery period, subjects performed an endurance performance trial on a computerized cycle ergometer (VeloTron, Racermate, Inc). The start of the trial consisted of 20 minutes at a steady-state pace, with intensity corresponding to 60% W_{\max} . The purpose of the steady state segment was to assess steady-state substrate utilization, and to ensure depletion of muscle glycogen. Following 20 minutes of steady-state exercise, subjects completed a simulated 20 km time trial. The last 5 km of the course consisted an uphill segment at 5% elevation. This was included in the protocol to allow an assessment of late-exercise performance.

All trials were performed at ambient room temperature (70-72 °F). The subjects were asked to void their bladders prior to all trials. A Windmere fan, set on ‘medium’ speed, was placed 2 meters from the handlebars of the ergometer for cooling purposes during the trial. Water was provided *ad libitum* throughout all trials. Each subject was asked to provide maximal effort and treat each time trial as a competitive event. For consistency, no verbal encouragement was provided to the subjects during the time trial. The only feedback subjects received during the trials regarding performance were distance remaining and distance completed.

Subjects were given at least one week between trials to ensure complete recovery. Participants were allotted 14 days to complete a trial in the event of unrelated illness or unforeseen circumstances, which occurred in four subjects.

Dependent measurements

Subsequent Exercise Performance

Exercise performance was measured using cycling time to completion for the 20 km time trial. Time was also recorded during the final 5km of the course.

Physiological Responses during Steady-State Exercise

Metabolic Measurements

Metabolic measurements were obtained during the glycogen-depleting trial and during the steady-state segment of the subsequent performance trial using a SensorMedics Spectra metabolic cart. These measurements were obtained after 3 minutes of cycling during the glycogen depleting trial (at 60% W_{\max}) and after 3 minutes of cycling during the subsequent performance trial (also at 60% W_{\max}). Expired respiratory gases were collected for 5 minutes at each time point. Average values were calculated using the final 3 minutes of each time period, following 2 minutes of breathing equilibration. Measurements obtained from the expired gases included oxygen uptake (VO_2), ventilation (VE), and respiratory exchange ratio (RER).

Blood Glucose and Lactic Acid

Blood samples were obtained immediately prior to exercise, during the glycogen-depleting trial (at minute 8) and exercise performance trial (at minute 8), and 30 minutes post-exercise. Approximately 0.5 ml of blood was collected

during each point via a finger-stick. An automated analyzer (YSI 2300 STAT glucose/lactate analyzer) determined glucose and lactate levels.

Serum Insulin

Thirty minutes after consuming the recovery beverage, 5 ml of blood was collected using a venous blood draw from an antecubital vein. The blood was collected in SST clot agent Vacutainer[®] tubes, centrifuged, and serum stored at -80 °C. Insulin levels were assessed from the serum through a commercially-available ELISA assay (ALPCO Diagnostics, Salam, NH).

Heart Rate

Heart rate was recorded using a Polar heart rate monitor (Lake Success, NY). Measurements were obtained at minute 8 of the glycogen-depleting trial and the subsequent exercise trial. Average heart was also recorded for the 20 km time trial and the final 5 km segment.

Ratings of Perceived Exertion (RPE)

Ratings of perceived exertion were obtained by having the subjects point to a corresponding level of exertion on the Borg RPE scale (6-20). The RPE recording was collected at the 8 minute mark during the glycogen-depleting and subsequent exercise. The following statement was read to the subjects to explain the RPE scale: *“Please describe your current level of exertion using the following scale. This level should represent your overall perception of effort, and not localized to a specific group of muscles, etc. For reference, a 6 would represent your effort when you are resting or watching TV, while 20 would represent the highest level of exertion you are capable of producing during exercise”*.

Muscle Recovery Variables

Muscle recovery variables included ratings of muscle soreness, mental and physical energy/fatigue ratings, isometric peak torque (MVC), and serum creatine kinase (CK). Muscle soreness ratings and mental/physical energy/fatigue ratings were collected during the following three time points; prior to the glycogen depletion trial (PRE), prior to the subsequent exercise performance trial (4HrPost) and 24 hours following the glycogen depletion trial (24HrPost). Serum CK was obtained twice: PRE and 24HrPost. Peak MVC was assessed five times: PRE, 4HrPost, 24HrPost, as well as immediately after the glycogen depleting trial and subsequent trial. A visual timeline of the muscle recovery measurements is provided in **Figure 4.1** above.

Serum Creatine Kinase (CK)

Serum CK was measured as an indirect indicator of muscle damage. Approximately 5ml of blood were obtained using a venous blood draw from an antecubital vein prior to exercise and 24-hr post exercise. Using a centrifuge, whole blood was spun at 7000 rpm to separate serum and stored in a -80 ° C freezer. Serum CK was analyzed using a Johnson and Johnson Vitro DT6011. The measurement device was calibrated prior to analyses using a reconstituted lyophilized calibration standard purchased from the manufacturer.

Muscle Function Test/Isometric peak torque (MVC)

Peak torque of the knee extensors were assessed to determine treatment differences in muscle function during recovery from exercise. Subjects were seated in a modified leg extension chair and asked to push as hard as possible against a shin pad that was connected to a force transducer. Each subject

performed 3 to 6, 5-second repetitions at each time-point. There was a 1 minute rest between each repetition. MVC was also assessed immediately following both exercise sessions.

Mental and Physical Energy/Fatigue Ratings

The Mental and Physical State and Trait Energy and Fatigue Scales (MPSTEFS, O’Conner, 2004) were used to record ratings of mental and physical energy. Separate ratings of physical energy, physical fatigue, mental energy and mental fatigue were obtained using visual analog scales, with potential scores ranging from 0 to 300 for each rating.

Muscle Soreness Ratings

During the familiarization trial subjects received instruction on how to utilize the muscle soreness scale. The following statement was read to the subjects; “please describe your overall level of muscle soreness that you feel currently while performing normal daily activities (i.e. walking up or down stairs)”. The soreness rating used a 100mm visual analog scale; 0 indicated no muscle soreness and 100 indicated impaired movement due to muscle soreness.

Genotypes

Single nucleotide polymorphisms (SNPs) were evaluated in this study to determine if specific variations in these polymorphisms were associated with variations in muscle recovery variables, and examine their influence on individual responses to recovery beverages with protein. The following SNPs were evaluated in this study: ACTN3 R577X, IGF-II C13790G, IGF-II G17200A, IGF2AS A1364C, and IGF2AS G11711T. Approximately 4 ml of blood were obtained using a venous blood draw from

an antecubital vein prior to the glycogen depleting exercise during the first trial, and mixed through inversion. DNA was isolated from each blood sample using the QIAamp® DNA Blood Mini Kit (Qiagen, CA). Subject identification information was removed for all DNA samples, which were subsequently shipped to the Research Center for Genetic Medicine at the Children's National Medical Center in Washington D.C. for genotyping. The following polymorphisms were selected for genotyping based on their susceptibility to impaired muscle recovery as demonstrated by increased serum CK levels, strength loss, and muscle soreness following eccentric resistance exercises in prior research:

- 1) IGF-II C13790G (homozygous rare allele found in 18.6% of subjects): 58% strength loss immediately post-exercise, increased serum CK activity at 7 days post-exercise (8818 U/l) (Devaney et al., 2007).
- 2) IGF-II G17200A (homozygous rare allele found in 11.0% of subjects): 60% strength loss immediately post-exercise, increased CK activity at 7 days post-exercise (12261 U/l) (Devaney et al., 2007).
- 3) IGF2AS A1364C (homozygous wild type found in 36.2% of subjects): 58% strength loss, 50mm soreness (Devaney et al., 2007).
- 4) IGF2AS G11711T (homozygous wild type found in 27.8% of subjects): 56% strength loss, 56mm soreness, increase in blood CK (17,160 U/l) and Mb (659 ng/ml) (Devaney et al., 2007).

In addition, ACTN3 R577X was selected for genotyping due to its potential for demonstrating improved endurance performance in homozygous rare allele subjects (Yang et al., 2003; Niemi and Majamäa, 2005; Lucia et al., 2006; and Eynon et al., 2009).

Genotyping was performed using Applied Biosystem's Taqman allele discrimination assay using standard thermal cycling conditions, with genotypes called by the 7900HT Real-Time PCR System (Applied Biosystems, CA). For all the SNPs, the Applied Biosystem assay ID is located in **Table 4.1** or if the SNP was not available on the ABI website than a custom primer set was designed.

Table 4.1: TaqMan primer sets for SNPs tested.

Gene	SNP/ rs number	Forward Primer or ABI Assay #	Reverse Primer	WT allele probe (5' VIC)	MT allele probe (5' FAM)
IGF-II	C13790G rs3213221	C__31456535_10			
IGF2AS	G11711T rs7924316	C_29192859_10			
IGF-II	G17200A rs680	TGGACTTGAGT CCCTGAACCA	GTGCCCCACCT GTGATTCTG	AGAGAAAA GAAGGGCC	AAAGAGAAA AGAAGGACC
IGF2AS	A1364C rs4244808	C__2981208_10			
ACTN3	R577X rs1815739	C____590093_1_			

Study Treatments

Recovery beverages were consumed immediately following the glycogen depleting exercise, 2 hours post glycogen depleting exercise, and immediately following the subsequent exercise performance trial. The study protocols were performed by each subject on three separate occasions (separated by 7-14 days each). Subjects consumed a different beverage for each trial. The beverages were provided in a randomly-counterbalanced, double-blinded design. Beverages were refrigerated at 5 °C from 2

hours prior to each trial until immediately prior to being consumed. Subjects were informed that the beverages consisted of the same amount of fluid with different nutrient composition, but were not informed the specific composition of the different beverages.

As this genotype study was conducted as part of a larger recovery beverage study, it adopted identical protocols that involved the administration of three different treatments. However, for the purposes of the current genotype study, only data collected from the carbohydrate-only treatment (CHO) and the high-carbohydrate low-protein treatment (HCLP) were analyzed.

Carbohydrate-only Beverage (CHO)

The CHO beverage contained 300 kcal, and 75g of carbohydrate, per 625 ml serving (12% by volume). To provide an isocaloric and isovolumetric (750 ml) comparison to the other beverage, the CHO beverage was consumed along with 125 ml of water.

High-Carbohydrate, Low-Protein Beverage (HCLP)

Each 250 ml serving of the HCLP beverage contained 300 kcal, 50g carbohydrate, 25g protein, and 0.5g of fat. To provide an isocaloric and isovolumetric (750 ml) comparison to the other beverage, the HCLP mixture was consumed along with 500 ml of water.

Dietary and Exercise Controls

Subjects were asked not to perform any heavy exercise 48 hours prior to each trial. A 48-hour diet and exercise log was completed by each subject preceding each treatment trial, and participants were instructed to maintain a consistent dietary and exercise habit between trials. No less than 12 hours prior to the start of the exercise,

subjects consumed their final self-selected meal (dinner the evening prior to testing). After this meal, subjects followed the standardized dietary protocol consisting of the following procedures:

- 1) No food or beverage intake between dinner and bedtime on the evening prior to the trial (water was consumed *ad libitum*).
- 2) On the morning of the exercise trials, subjects consumed a standardized breakfast two hours prior to the glycogen-depleting exercise trial. The meal was provided by the researchers, and consisted of 495-510 kcal, including 90-98g carbohydrate, 8-12g protein and 4.5-7.5g fat. In order to account for personal tastes, subjects chose from one of 4 meal choices, but the identical meal chosen was repeated across the four trials for each subject. These dietary restrictions ensured that no differences in nutrient intake occurred between the intervention periods, other than the treatments provided by the researchers.
- 3) No nutrients were consumed between the glycogen depleting trial and the subsequent exercise performance trial, other than the recovery beverages provided by the researchers.
- 4) No nutrients were consumed for 2 hours following the subsequent exercise performance trial, other than the recovery beverages provided by the researchers.
- 5) Subjects resumed their normal diets from 2 hours following the subsequent exercise performance trial through the 24HrPost measurement period. However, subjects recorded their dietary intake during this period, and were instructed to maintain similar dietary habits between the different trials.

Statistical Analyses

SPSS Version 17.0 was used to conduct statistical analyses. One-way Analysis of Variance (ANOVA) was used to analyze main effects for allele groups for time to

completion for the 20 km and final 5 km during subsequent exercise performance. This analysis was repeated for each of the five selected genotypes. Pairwise comparisons were performed to analyze specific differences in performance variables between allele groups for each genotype. To analyze genotype effects on markers of muscle recovery (serum CK levels, peak MVC, mental and physical energy/fatigue and muscle soreness ratings), repeated measures ANOVAs (RMANOVA) were conducted with 'time' as the within-subject factor (pre, post) and 'allele group' as the between-group factor. Post-hoc analyses were conducted using pairwise comparisons to analyze specific differences in change scores for each recovery variable between allele groups for each genotype. For specific allele groups identified as susceptible to impaired muscle recovery, RMANOVAs were conducted within the genotype group, with treatment (CHO, HCLP) and time (pre, post) as within-subject factors.

Results

Demographics

The sample population demographics (age, height, body mass, peak aerobic capacity, and power output) of the 12 trained male cyclists from which viable DNA could be extracted are presented in **Table 4.2**. The average $\text{VO}_{2\text{peak}}$ of $65.5 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ represents the 99th percentile for maximal aerobic power based on age-specific norms prescribed by ACSM (2010).

Table 4.2: Subject Demographics.

Variable	Mean \pm SEM
Age (y)	25.5 ± 2.3
Height (cm)	177.5 ± 2.2
Weight (kg)	73.3 ± 2.3
$\text{VO}_{2\text{peak}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	65.5 ± 2.1
W_{max} (W)	351.7 ± 9.8

Genotypes

The genotype distributions of the sample population are presented in **Table 4.3**. They include genotype and allele frequencies for ACTN3 SNPs, IGF-II SNPs, and IGF2AS SNPs. All 12 subjects were homozygous for the wild-type allele of IGF-II ApaI (G17200A). Hence, analyses of genotype variations on subsequent performance and markers of recovery were not performed for the IGF-II ApaI G17200A SNP.

Table 4.3: Genotype Distributions and Frequencies.

Gene	SNP/(rs number)	Homozygous Wild Type	Heterozygous	Homozygous Rare Allele	Allele Frequency Wild type/Rare
ACTN3	R577X (rs1815739)	2 (16.7%, RR)	8 (66.7%, RX)	2 (16.7%, XX)	0.500/0.500
IGF-II	C13790G (rs3213221)	6 (50%, CC)	6 (50%, CG)	0 (0%, GG)	0.750/0.250
IGF-II	G17200A (rs680)	12 (100%, GG)	0 (0%, GA)	0 (0%, AA)	1.000/0.000
IGF2AS	A1364C (rs4244808)	2 (16.7%, AA)	5 (41.7%, AC)	5 (41.7%, CC)	0.375/0.625
IGF2AS	G11711T (rs7924316)	2 (16.7%, GG)	7 (58.3%, GT)	3 (25.0% TT)	0.458/0.542

Subsequent Performance

Performance variables for the subsequent exercise trial (time for 20 km and final 5 km) are presented in **Table 4.4**. The CHO treatment served as the ‘control’ trial. Thus, all analyses of the influence of genotype on exercise/performance were performed using data from the CHO trial.

Table 4.4: Subsequent Exercise Performance – 20 km Time Trial (CHO Treatment).

Variable	Mean \pm SEM
Total time to completion for 20 km (min)	48.5 \pm 1.5
Time for final 5 km climb (min)	19.6 \pm 0.9

Genotype effects on subsequent performance were examined for the CHO treatment, and the findings are presented in **Table 4.5**. Subjects homozygous for the wild-type allele of IGF2AS G11711T demonstrated significantly slower time to completion for the 20 km time trial ($P < 0.05$), as well as significantly slower time for the final 5 km climb ($P < 0.05$). Subjects homozygous for the rare allele of ACTN3 R577X exhibited a potential trend towards faster ride times in the final 5 km climb ($P < 0.10$). However, the difference was not statistically significant ($P > 0.05$). No significant genotype effects were observed for any other genotypes on subsequent exercise performance.

Table 4.5: Genotype and Subsequent Exercise Ride Times for CHO treatment.

Variable	Genotype/SNP/ (rs number)	Allele/Subjects (n)	Mean \pm SEM
20 km time (min)	IGF2AS G11711T* (rs7924316)	Homozygous Wild Type (GG, n = 2)**	55.8 \pm 1.1
		Heterozygous Wild Type (GT, n = 7)	47.3 \pm 1.9
		Homozygous Rare Allele (TT, n = 3)	46.5 \pm 1.2
	IGF2AS A1364C (rs4244808)	Homozygous Wild Type (AA, n = 2)	45.5 \pm 1.0
		Heterozygous Wild Type (AC, n = 5)	49.3 \pm 1.9
		Homozygous Rare-Allele (CC, n = 5)	48.9 \pm 3.9

5 km climb (min)	IGF-II C13790G (rs3213221)	Homozygous Wild Type (CC, n = 6)	47.8 \pm 1.5
		Heterozygous Wild Type (CG, n = 6)	49.2 \pm 2.7
	ACTN3 R577X (rs1815739)	Homozygous Wild Type (RR, n = 2)	50.9 \pm 6.0
		Heterozygous Wild Type (RX, n = 8)	49.2 \pm 1.5
		Homozygous Rare Allele (XX, n = 2)	43.3 \pm 3.3
	IGF2AS G11711T [#] (rs7924316)	Homozygous Wild Type (GG, n = 2)**	24.5 \pm 1.4
		Heterozygous Wild Type (GT, n = 7)	18.7 \pm 1.1
		Homozygous Rare Allele (TT, n = 3)	18.7 \pm 0.7
	IGF2AS A1364C (rs4244808)	Homozygous Wild Type (AA, n = 2)	18.1 \pm 0.7
		Heterozygous Wild Type (AC, n = 5)	19.8 \pm 1.0
		Homozygous Rare Allele (CC, n = 5)	20.1 \pm 2.1
	IGF-II C13790G (rs3213221)	Homozygous Wild Type (CC, n = 6)	19.5 \pm 0.8
		Heterozygous Wild Type (GC, n = 6)	19.8 \pm 1.7
	ACTN3 R577X (rs1815739)	Homozygous Wild Type (RR, n = 2)	22.1 \pm 3.8
		Heterozygous Wild Type (RX, n = 8)	19.9 \pm 0.8
		Homozygous Rare Allele (XX, n = 2) [^]	16.4 \pm 3.4

* signifies a trend towards a difference ($P < 0.10$)

[#] significantly different from each other ($P < 0.05$)

** GG times were significantly slower ($P < 0.05$) than GT and TT

[^] signifies a trend towards faster times for XX than RR ($P < 0.10$)

Based on the above findings in the CHO control trial, the genotypes that were most likely to be associated with differences in subsequent performance were compared against the HCLP trial to determine if possible treatment effects between CHO and HCLP were influenced by genotypes. There were no significant treatment x genotype interactions for 20 km and final 5 km times (**Table 4.6**).

Table 4.6: Treatment by Genotype interaction in Subsequent Exercise.

Variable	Genotype/SNP/(rs)	Treatment	P Value (treatment x genotype interaction)
20 km time (min)	IGF2AS G11711T (rs7924316)	CHO	0.980
		HCLP	
	ACTN3 R577X (rs1815739)	CHO	0.258
		HCLP	
5 km climb (min)	IGF2AS G11711T (rs7924316)	CHO	0.941
		HCLP	
	ACTN3 R577X (rs1815739)	CHO	0.325
		HCLP	

Recovery Variables

The markers of muscle recovery following the glycogen depleting exercise were examined for the CHO treatment, and findings are presented in **Table 4.7**. There was a small but significant increase in CK from pre-exercise to 24-hours post exercise (25.0 U/L, $P < 0.05$). Peak muscle function (MVC) trended towards higher levels 24-hours post-exercise ($P < 0.10$), and tended to be lower immediately following glycogen depleting exercise, but these tendencies were not statistically significant ($P > 0.05$). There were significant declines ($P < 0.05$) in physical energy and mental energy ratings, and significant increases ($P < 0.05$) in physical fatigue and mental fatigue ratings from pre-exercise to 4-hours post exercise. No significant differences were observed in any of the above ratings from pre-exercise to 24-hours post exercise. Muscle soreness ratings were significantly elevated ($P < 0.05$) from pre-exercise to 4-hours post exercise, and from pre-exercise to 24-hours post exercise.

Table 4.7: Markers of Recovery for CHO Treatment.

Variables	Time Point	Mean \pm SEM
Creatine Kinase (U/L)	PreGE	136.2 \pm 12.0
	24Post	161.1 \pm 17.0*
Muscle Function (MVC)	PreGE	451.1 \pm 29.9
	0Post	427.2 \pm 41.2
	4Post	445.9 \pm 24.8
	24Post	489.5 \pm 34.7*
Muscle Soreness (mm)	PreGE	10.5 \pm 3.2
	4Post	36.0 \pm 5.2*
	24Post	24.9 \pm 6.5*
Physical Energy (mm)	PreGE	200.3 \pm 18.2
	4Post	159.7 \pm 17.5**
	24Post	200.4 \pm 18.6

Physical Fatigue (mm)	PreGE	79.2 \pm 16.9
	4Post	148.1 \pm 19.0*
	24Post	87.5 \pm 17.6
Mental Energy (mm)	PreGE	194.7 \pm 20.3
	4Post	174.6 \pm 17.8**
	24Post	200.6 \pm 19.2
Mental Fatigue (mm)	PreGE	94.4 \pm 19.4
	4Post	117.5 \pm 17.6*
	24Post	89.3 \pm 20.1

PreGE = pre glycogen depleting exercise, 0Post = 0 hours post glycogen depleting exercise, 4Post = 4 hours post glycogen depleting exercise, 24Post = 24 hours post glycogen depleting exercise.

* significant increase from PreGE ($P < 0.05$)

** significant decline from PreGE ($P < 0.05$)

The change scores of muscle damage/recovery variables for the CHO treatment (control trial) were analyzed for genotype effects, and significant findings are presented in **Table 4.8**.

Subjects homozygous for the wild-type allele of ACTN3 R577X demonstrated significantly greater losses in peak MVC from pre to 4-hours post exercise than heterozygous subjects ($P < 0.05$). The loss of peak muscle function in the wild-type allele group also tended to be larger, (but not significantly so) when compared to subjects homozygous for the rare allele of ACTN3 R577X ($P > 0.05$). Subjects homozygous for the rare allele of IGF2AS A1364C demonstrated significantly greater declines in peak MVC from pre to 4-hours post exercise compared to subjects homozygous for the wild allele ($P < 0.05$). When genotyped for IGF-II C13790G, heterozygous subjects demonstrated significantly greater declines in peak MVC than subjects homozygous for the wild-type allele ($P < 0.05$). There were no genotype effects observed for changes in peak MVC between 0-hour and 24-hours post exercise.

When genotyped for the IGF2AS G11711T SNP, significant differences ($P < 0.05$) were observed between genotypes for changes in physical fatigue ratings from pre-exercise to 4-hours and 24-hours post exercise, and for changes in mental fatigue ratings from pre-exercise to 24-hours post exercise (see **Table 4.8**). When genotyped for ACTN3 R577X, changes in physical energy ratings were significantly different between genotypes from pre-exercise to 4-hours post-exercise ($P < 0.05$), and a trend towards significance from pre-exercise to 24-hours post exercise ($P < 0.10$). There were no genotype effects observed for changes in physical/mental energy/fatigue ratings at any time point when subjects were genotyped for IGF-II C13790G and IGF2AS A1364C.

Changes in muscle soreness ratings demonstrated a trend towards significance from pre-exercise to 24-hours post exercise when genotyped for IGF 2AS G11711T. There were no significant genotype effects observed for changes in serum CK responses.

Table 4.8: Significant Associations of Genotypes with Markers of Muscle Recovery.

Gene/SNP/(rs number)/ Variable	Time points	P Value (genotype 'main effects')	Change scores ¹ \pm SE
ACTN3 R577X (rs1815739)			
Peak Muscle Function	PreGE – 4Post	0.070 [#]	RR (n = 2, 66.3 \pm 40.4)* RX (n = 8, -11.9 \pm 10.4)* XX (n = 2, 12.5 \pm 38.7)
Physical Energy ratings	PreGE – 4Post	0.019*	RR (n = 2, 78.8 \pm 15.8) RX (n = 8, 20.6 \pm 10.0)** XX (n = 2, 82.5 \pm 18.5)
Physical Energy ratings	PreGE – 24Post	0.098 [#]	RR (n = 2, 40.5 \pm 35.0)* [#] RX (n = 8, -8.4 \pm 7.7)* XX (n = 2, -7.8 \pm 5.3) [#]
Mental Energy ratings	PreGE – 24Post	0.126	RR (n = 2, 33.8 \pm 70.8)* RX (n = 8, -9.8 \pm 13.0)* XX (n = 2, -25.5 \pm 3.5)
IGF-II C13790G (rs3213221)			
Peak Muscle Function	PreGE – 4Post	0.018*	CC (n = 6, -23.5 \pm 11.6)* CG (n = 6, 33.8 \pm 16.6)*

IGF2AS A1364C (rs4244808)			
Peak Muscle Function	PreGE – 4Post	0.086 [#]	AA (n = 2, -49.7 ± 23.6)* AC (n = 5, 1.6 ± 7.8) CC (n = 5, 30.8 ± 23.0)*
IGF2AS G11711T (rs7924316)			
Physical Energy ratings	PreGE – 24Post	0.167	GG (n = 2, 33.0 ± 42.5) [#] GT (n = 7, -11.8 ± 8.2) [#] TT (n = 3, 5.0 ± 5.0)
Mental Energy ratings	PreGE – 4Post	0.093 [#]	GG (n = 2, 12.8 ± 3.8) GT (n = 7, 34.3 ± 8.0)* TT (n = 3, -8.3 ± 22.3)*
Mental Energy ratings	PreGE – 24Post	0.102	GG (n = 2, 52.0 ± 57.5) [#] GT (n = 7, -17.9 ± 9.1)* TT (n = 3, -16.7 ± 18.8) [#]
Physical Fatigue ratings	PreGE – 4Post	0.022*	GG (n = 2, 120.5 ± 9.0)* GT (n = 7, 72.0 ± 8.9) [#] TT (n = 3, 27.3 ± 26.9) [#]
Physical Fatigue ratings	PreGE – 24Post	0.001*	GG (n = 2, 98.3 ± 21.3)** GT (n = 7, -14.8 ± 9.6) TT (n = 3, 2.2 ± 4.7)
Mental Fatigue ratings	PreGE – 4Post	0.174	GG (n = 2, 36.8 ± 18.3) GT (n = 7, 32.9 ± 11.1) [#] TT (n = 3, -9.0 ± 21.9) [#]
Mental Fatigue ratings	PreGE – 24Post	0.008*	GG (n = 2, 75.3 ± 29.3)** GT (n = 7, -20.8 ± 9.0) TT (n = 3, -22.3 ± 21.9)
Muscle Soreness ratings	PreGE – 24Post	0.189	GG (n = 2, 36.8 ± 19.8) [#] GT (n = 7, 11.9 ± 6.9) TT (n = 3, 5.5 ± 3.6) [#]

PreGE = pre glycogen depleting exercise, 4Post = 4 hours post glycogen depleting exercise, 24Post = 24 hours post glycogen depleting exercise.

¹Larger change scores in peak muscle function, physical/mental energy ratings represent greater declines in the respective variables, while larger change scores in physical/mental fatigue and muscle soreness ratings represent greater increases in those variables.

* significantly different from each other (P < 0.05)

** significantly different than the other two groups (P < 0.05)

[#] signifies a trend towards a difference (P < 0.10)

Based on the above findings in the CHO control trial, the genotypes that were associated with differences in the change scores for muscle recovery variables were

compared against the HCLP trial to examine whether genotypes influenced potential treatment effects on recovery measurements. A summary of these treatment x time x genotype analyses are shown in **Table 4.9**.

Table 4.9: Treatment x time within Genotypes on markers of muscle recovery.

Gene/SNP/(rs number)/Variable	Time points	Treatment	P Value (treatment effect)
ACTN3 R577X (rs1815739)			
Peak Muscle Function	PreGE – 4Post	CHO HCLP	0.083 [#]
Physical Energy ratings	PreGE – 4Post	CHO HCLP	0.960
Physical Energy ratings	PreGE – 24Post	CHO HCLP	0.135
Mental Energy ratings	PreGE – 24Post	CHO HCLP	0.113
IGF-II C13790G (rs3213221)			
Peak Muscle Function	PreGE – 4Post	CHO HCLP	0.242
IGF2AS A1364C (rs4244808)			
Peak Muscle Function	PreGE – 4Post	CHO HCLP	0.083 [#]
IGF2AS G11711T (rs7924316)			
Physical Energy ratings	PreGE – 24Post	CHO HCLP	0.920
Mental Energy ratings	PreGE – 4Post	CHO HCLP	0.008*
Mental Energy ratings	PreGE – 24Post	CHO HCLP	0.333
Physical Fatigue ratings	PreGE – 4Post	CHO HCLP	0.232
Physical Fatigue ratings	PreGE – 24Post	CHO HCLP	0.136
Mental Fatigue ratings	PreGE – 4Post	CHO HCLP	0.146

Mental Fatigue ratings	PreGE – 24Post	CHO HCLP	0.123
Muscle Soreness ratings	PreGE – 24Post	CHO HCLP	0.031*

* significantly different from each other ($P < 0.05$)

signifies a trend towards a difference ($P < 0.10$)

Treatment x time analyses were performed within the genotype groups that were identified to be susceptible to impaired muscle recovery following heavy endurance exercise (**Table 4.10**). Small sample-sizes ($n = 2 - 6$) precluded any strong likelihood to observe statistically significant differences between treatments. However, visual analysis of the means showed that markers of muscle recovery improved with the HCLP treatment in nine of the fourteen variables examined (**Table 4.10**). Three variables were identical between treatments and two variables showed worsened scores with the HCLP treatment.

Table 4.10: Treatment x Time Effects on markers of muscle recovery within “susceptible” Genotype groups.

Gene/SNP/(rs number)/ Allele/Variable	Treatment	Time points	Mean \pm SEM	P Value (treatment x time effect)
ACTN3 R577X (rs1815739) Homozygous Wild-Type (RR, $n = 2$)				
Peak Muscle Function	CHO	PreGE	588.5 \pm 165.7	0.429*
		4Post	522.2 \pm 125.3	
	HCLP	PreGE	565.3 \pm 119.1	
		4Post	603.2 \pm 161.9	
Physical Energy ratings	CHO	PreGE	180.5 \pm 84.5	0.972
		4Post	101.8 \pm 68.8	
	HCLP	PreGE	181.0 \pm 105.0	
		4Post	105.0 \pm 27.0	
Physical Energy ratings	CHO	PreGE	180.5 \pm 84.5	0.612*
		24Post	140.0 \pm 49.5	
	HCLP	PreGE	181.0 \pm 105.0	
		24Post	156.8 \pm 93.3	
Mental Energy ratings	CHO	PreGE	149.8 \pm 72.3	0.587*
		24Post	100.3 \pm 12.3	
	HCLP	PreGE	171.0 \pm 68.0	
		24Post	178.3 \pm 82.8	

IGF-II C13790G (rs3213221) Heterozygous Wild-Type (CG, n = 6)				
Peak Muscle Function	CHO	PreGE	484.4 ± 56.2	0.449*
		4Post	450.6 ± 43.4	
	HCLP	PreGE	483.0 ± 40.3	
		4Post	476.7 ± 58.5	
IGF2AS A1364C (rs4244808) Homozygous Rare Allele (CC, n = 2)				
Peak Muscle Function	CHO	PreGE	484.6 ± 67.6	0.420*
		4Post	453.8 ± 49.4	
	HCLP	PreGE	522.8 ± 51.9	
		4Post	527.7 ± 65.6	
IGF2AS G11711T (rs7924316) Homozygous Wild-Type (GG, n = 2)				
Physical Energy ratings	CHO	PreGE	193.8 ± 71.3	0.857
		24Post	160.8 ± 28.8	
	HCLP	PreGE	214.0 ± 72.0	
		24Post	169.3 ± 80.8	
Mental Energy ratings	CHO	PreGE	172.3 ± 49.8	0.327 [#]
		4Post	159.5 ± 46.0	
	HCLP	PreGE	186.5 ± 52.5	
		4Post	92.3 ± 2.8	
Mental Energy ratings	CHO	PreGE	172.3 ± 49.8	0.699*
		24Post	120.3 ± 7.8	
	HCLP	PreGE	186.5 ± 52.5	
		24Post	179.0 ± 82.0	
Physical Fatigue ratings	CHO	PreGE	52.5 ± 31.5	0.984
		4Post	173.0 ± 22.5	
	HCLP	PreGE	56.0 ± 45.0	
		4Post	175.0 ± 23.0	
Physical Fatigue ratings	CHO	PreGE	52.5 ± 31.5	0.478*
		24Post	150.8 ± 10.3	
	HCLP	PreGE	56 ± 45.0	
		24Post	124.5 ± 51.5	
Mental Fatigue ratings	CHO	PreGE	94.5 ± 2.5	0.576 [#]
		4Post	131.3 ± 20.8	
	HCLP	PreGE	76.5 ± 16.5	
		4Post	177.3 ± 46.8	

Mental Fatigue ratings	CHO	PreGE	94.5 ± 2.5	0.521*
		24Post	169.8 ± 26.8	
	HCLP	PreGE	76.5 ± 16.5	
		24Post	84.8 ± 58.8	
Muscle Soreness ratings	CHO	PreGE	17.5 ± 15.5	0.194*
		24Post	54.3 ± 4.3	
	HCLP	PreGE	14.0 ± 11.0	
		24Post	24.5 ± 5.0	

* improvements observed with HCLP treatment

declines observed with HCLP treatment

Discussion

The genotype distributions and allele frequencies of the ACTN3 R577X, IGF-II C13790G, and IGF2AS G11711T SNPs in the sample population are similar to those reported by Clarkson et al. (2005) and Devaney et al. (2007). The population frequency of the IGF-II G17200A and IGF2AS A1364C SNPs differed from the findings of Devaney et al. In our study, subjects were exclusively homozygous for the wild-type allele of IGF-II G17200A, and there were more subjects homozygous for the rare allele than the wild-type allele of IGF2AS A1364C. These discrepancies could be attributed to a small sample size ($n = 12$) and possibly an all-Caucasian sample in the present study, compared to a larger and more diverse sample population analyzed by Devaney et al (J M Devaney, personal communication, April 2, 2010).

The most important findings in this study were that the ACTN3 R577X, IGF-II C13790C, IGF2AS A1364C, and IGF2AS G11711T SNPs were associated with alterations in markers of muscle recovery following heavy endurance exercise. In addition, ACTN3 R577X and IGF2AS G11711T SNPs were observed to be potentially associated with subsequent aerobic exercise performance.

Subjects homozygous for the wild-type allele of IGF2AS G11711T SNP had significantly slower times to completion during subsequent exercise for the 20 km (55.8 ± 1.1 min) and final 5 km (24.5 ± 1.4 min) in the CHO control trial than heterozygous or homozygous rare allele subjects. This could be attributed to a greater impairment of muscle recovery, as demonstrated by a substantial increase in physical fatigue ratings (120.5 ± 9.0 mm), and an increase in mental fatigue ratings (36.8 ± 18.3 mm) from pre to 4-hours post glycogen depleting exercise within this allele group. These results agreed

with the findings of Devaney et al., who reported impaired muscle function following eccentric resistance exercise in homozygous wild-type subjects (2007). Alternatively, this genotype could have influenced the general performance capacity of the individual subjects, rather than recovery *per se*. This possibility is supported by the observation that homozygous wild-type subjects completed only 42.3 ± 7.3 min in their glycogen depleting trial, compared to >60 min in the other genotype groups.

Subjects homozygous for the rare allele of ACTN3 R577X trended towards faster ride times for the final 5 km (16.4 ± 3.4 min) in the CHO control trial than heterozygous and homozygous wild-type subjects ($P < 0.10$). Faster ride times were also recorded for rare allele subjects during the 20 km ride (43.3 ± 3.3 min), but they were not significantly different ($P > 0.05$) than the other allele groups. The expression of ACTN3 has been discovered to be limited to a subset of Type II fast twitch muscle fibers (North and Beggs, 1996). The 577X polymorphism results in deficiency of the alpha-actinin-3 protein, and this mutation may manifest more commonly in certain populations. Findings from Yang et al. (2003), Niemi and Majamäa (2005), Lucia et al. (2006), and Eynon et al. (2009) revealed a higher frequency of the XX (ACTN3 null) rare allele in endurance athletes (distance runners and elite cyclists), compared to a higher frequency of the RR allele in elite sprinters. In addition, Vincent et al. observed significantly greater distributions of Type IIX fibers in RR than XX-allele subjects (2007). These findings suggest that the R allele may be more associated with anaerobic sprint performance, whereas the X allele may be more associated with endurance performance. Hence, while the time to completion may not be significantly different in our study, the results agreed with previous studies as faster riding times were observed in XX-allele subjects. This also

supports our previous statement that the genotype may be influencing general performance capacity more than ‘recovery’.

There were no significant changes in CK responses from pre-exercise to 24-hours post exercise during the CHO control trial in all genotype groups. A possible explanation for the lack of significant CK elevations with exercise may be attributed to the training status of the sample population in this study. The average $\text{VO}_{2\text{peak}}$ of the 12 subjects in this study was $65.5 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, which represents the 99th percentile for maximal aerobic power based on age-specific norms prescribed by ACSM (2010). This was higher than the $\text{VO}_{2\text{peak}/\text{max}}$ values observed in prior cycling studies conducted by our laboratory (Saunders, Kane, and Todd, 2004; Romano-Ely et al., 2006; Saunders, Luden, and Herrick, 2007; Valentine et al., 2008; and Saunders et al., 2009). The higher maximal aerobic capacity observed in our present study may indicate that the intensity/workload prescribed in the glycogen depleting exercise did not provide a stimulus challenging enough to impose substantial increases in muscle damage, as measured by serum CK responses. Consequently, this impaired our ability to discern the potential effects of genotype on variations in CK responses post-exercise.

Changes in peak muscle function (MVC) measured 4-hours post exercise were observed to be more sensitive to significant changes from pre-glycogen exercise levels compared to 24-hours post exercise, when they had returned to pre-exercise levels. Subjects homozygous for the wild-type allele of ACTN3 R577X sustained significantly greater strength losses ($66.3 \pm 40.4 \text{ N}$) 4-hours post exercise than rare allele subjects. The increased loss of strength in homozygous wild-type subjects may contribute to slower riding times than rare allele subjects, as described above. Conversely, observed strength

losses in this study differed from the findings of Clarkson et al. (2005), who reported that the ACTN3 R577X SNP was not associated with changes in strength post exercise. However, Clarkson et al. utilized an eccentric resistance exercise protocol to elicit muscle damage, and did not assess strength changes at 4-hours post exercise. One possible factor for the increased strength loss in homozygous wild-type subjects may be attributed to fiber type characteristics. Vincent et al. (2007) observed that the RR-allele is associated with increased distribution of Type IIX fibers (5%, $P < 0.05$). An increased recruitment of these fibers during the high-intensity interval segments of the glycogen depleting protocol may potentially elicit greater disruption to the sarcomere structures (e.g. Z-line streaming), which would lead to greater declines in MVC 4-hours post exercise. However, this development seems unlikely in our study, as it should have also resulted in increased CK levels.

Subjects heterozygous for the IGF-II C13790G SNP demonstrated significantly greater declines in peak MVC (33.8 ± 16.6 N) than subjects homozygous for the wild-type allele. Although our sample size was not large enough to include subjects homozygous for the rare allele, the results agreed with findings from Devaney et al. (2007), who found that the homozygous rare-allele subjects experienced significantly greater strength losses (58%) than homozygous wild-type subjects. This would indicate that the G allele may be associated with an increased loss of strength with exertional muscle damage, regardless of whether resistance or aerobic exercise is performed.

When genotyped for the IGF2AS A1364C SNP, homozygous rare allele subjects demonstrated significantly greater strength losses (30.8 ± 23.0 N) than homozygous wild-type allele subjects. These results differ from those of Devaney et al., who reported the

greatest strength loss (58%) in homozygous wild-type subjects. The differences may be attributed to our smaller population sample, compared to a much larger population sample ($n = 73$) assessed by Devaney et al. Our population was also exclusive to Caucasian males, whereas Devaney et al. did not classify their findings according to ancestry. A study conducted by the Devine Laboratory at the University of Maryland School of Medicine (Institute for Genome Sciences) as part of the International HapMap project examined the genotype distribution of 118 Utah residents of Northern and Western European ancestry (NCBI, 2003). When genotyped for the IGF2AS A1364C SNP, it was observed that 32.2% of the subjects were homozygous for the wild-type allele, 44.1% were heterozygous, and 23.7% were homozygous for the rare allele. The genotype distributions for this SNP were more closely matched by those reported in Devaney et al. (36.2%, 44.7%, 19.1%) than findings in the present study (16.7%, 41.7%, 41.7%) even though Devaney et al. did not genotype the sample population according to ancestry. This may indicate that ancestral variations in this genotype do not significantly impact the variables assessed in the present study. Hence, the differences in strength losses observed between the present study and those of Devaney et al. may be more related to the discrepancies in sample size.

Physical and mental energy and fatigue ratings, and muscle soreness ratings, were observed to be most sensitive to significant changes from pre-glycogen depleting exercise to 24-hours post exercise in the CHO control trial, although significant changes were observed in several ratings 4-hours post exercise as well. Subjects homozygous for the ACTN R577X wild-type allele reported significantly larger declines in physical and mental energy ratings (40.5 ± 35.0 mm, 33.8 ± 70.8 mm) 24 hours after their initial

exercise. This coincided with the above finding of decreased peak muscle function in these subjects, which would indicate that the RR wild-type allele of the ACTN3 R577X SNP may be more associated with impaired recovery from heavy endurance exercise.

The IGF2AS G11711T SNP appeared to have the greatest influence on physical/mental energy/fatigue and muscle soreness ratings among the genotypes analyzed. In particular, 24-hours after the initial exercise, subjects homozygous for the wild-type allele reported significantly larger declines in physical and mental energy ratings (33.0 ± 42.5 mm, 52.0 ± 57.5 mm), in conjunction with substantially larger elevations in physical and mental fatigue ratings (98.3 ± 21.3 mm, 75.3 ± 29.3 mm), as well as greater increases in muscle soreness ratings (36.8 ± 19.8 mm). In addition, as discussed above, homozygous wild-type subjects reported larger increases in physical and mental fatigue ratings 4-hours post glycogen depleting exercise, which might have contributed to their slower ride times during the subsequent exercise for the 20 km and final 5 km. These findings would suggest that the GG wild-type allele of the IGF2AS G11711T SNP may be more associated with impaired recovery from heavy endurance exercise, which would agree with the increased strength loss (58%) and soreness (56 mm) reported by Devaney et al. (2007).

A secondary purpose of the present study was to determine if potentially susceptible genotype groups (i.e. those expressing impaired muscle recovery) derived greater benefits from carbohydrate and protein ingestion, which have been shown in some studies to attenuate post-exercise disruptions in CK, muscle function, and muscle soreness. Treatment x time effects were examined for the alleles identified to be susceptible to impaired recovery, and several tendencies were observed for markers of

muscle recovery to be improved with the HCLP treatment. Peak muscle function was higher 4-hours post exercise during the HCLP trial than the CHO trial in all genotypes analyzed. Furthermore, six out of seven 24-hours post exercise ratings were found to be improved with the HCLP trial, with only the physical energy rating showing no changes between treatments in subjects homozygous for the IGF2AS G11711T wild-type allele. However, none of these treatment x time interactions were statistically significant, perhaps due to the small sample size for the genotype groups (n = 2-6). Hence, although our findings were preliminary in nature, they warrant further investigations with larger population samples to better understand the influence of genotype variations on response to carbohydrate-protein supplementation.

To summarize our findings, when assessing subsequent performance variables, we observed that the homozygous XX rare allele for ACTN3 R577X to be associated with improved performance during subsequent exercise, whereas the homozygous GG wild-type allele for IGF2AS G11711T was associated with slower riding times to completion. With regards to recovery variables, we observed that the homozygous RR wild-type allele for ACTN3 R577X, the homozygous GG rare allele for IGF-II C13790G, the homozygous CC rare allele for IGF2AS A1364C, and the homozygous wild-type GG allele for IGF2AS G11711T to be most susceptible to impaired recovery following heavy endurance exercise. Peak muscle function appeared to be most sensitive to significant changes from pre to 4-hours post glycogen depleting exercise, whereas physical/mental energy/fatigue and muscle soreness ratings appeared to be most sensitive to significant changes from pre to 24-hours post exercise. When assessing the treatment x time effects

within genotypes, the HCLP treatment demonstrated a potential tendency to improve markers of muscle recovery in ‘susceptible’ genotype groups.

We recommend that future studies recruit larger population samples to analyze treatment differences in subjects identified with the specific allele distributions listed above, and to incorporate an initial exercise protocol that elicits greater muscle damage.

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Chapter V

Conclusions

- 1) Genotype variations among subjects led to significant differences in peak muscle function (MVC) changes from resting levels to 4-hours following acute heavy endurance exercise.
- 2) Genotypes variations among subjects led to significant differences in changes for physical and mental energy and fatigue ratings, as well as changes in muscle soreness ratings, from resting levels to 24-hours following acute heavy endurance exercise.
- 3) Subjects with genotypes that were associated with greater increases in the above markers of muscle recovery tended to improve these variables with the ingestion of high carbohydrate – low protein beverages, although the differences were not statistically significant.

Appendix I

James Madison University School of Kinesiology and Recreation Studies Consent for Investigative Procedure

I, _____, hereby agree on _____ (date) to participate in the research project conducted by Dr. Michael Saunders, Dr. Nick Luden, Chris Boop and Qing Goh from James Madison University entitled "Altered carbohydrate and protein content in sports beverages: Influence on recovery from heavy endurance exercise".

Purpose

The purpose of this study is to determine if the ingredients in sports beverages affects short-term recovery from heavy cycling exercise. The proposed study will take approximately 4-5 weeks to complete. It will consist of a pretest physical assessment, a familiarization trial, and three treatment beverage/recovery trials (detailed below). The study will take place September, 2009 – August 2010, in the Human Performance Laboratory in Godwin Hall, Room 209, JMU.

Subject Responsibility

I understand that I will undergo the following testing in the study:

Pre-testing Phase:

Before any physical evaluation is given, I will complete pre-screening forms to insure that I meet the study criteria, and that I do not have any risk factors for heavy exercise. In the process of filling out these forms, I will share information regarding my general health and lifestyle with the researchers. In addition, I will notify the researchers if I have any known allergies to milk or milk-products. If I meet the criteria for the study, I will complete an assessment of my cardiorespiratory fitness and body mass. During this assessment, I will perform an exercise test to determine my maximal oxygen uptake (VO_{2max}). To do this, I will ride a stationary cycle at an initial workload that is 'fairly easy'. Workload will be increased every few minutes during the test. I will be encouraged to continue to cycle until I request to stop due to fatigue or am unable to continue at a cadence >60 rpm. In order to be included as a participant in the study, I must achieve a VO_{2max} of >45 ml/kg/min. If I meet these criteria, I will complete a familiarization trial, in which I perform the procedures described below (in *Treatment Phases*), without any of the testing that follows the *Subsequent Exercise Performance Test*.

Treatment Beverage/Recovery Trials

Following pre-testing, I will perform three exercise/recovery phases. Each of these phases will be identical, with the exception that a different sports beverage will be utilized during each phase. Approximately 7-14 days will be provided between each treatment phase.

Exercise/Recovery Phases:

Two hours following a standardized meal (see below), I will report to the laboratory to complete an exercise session on an exercise cycle, consisting of *high-intensity aerobic intervals*. This trial will take approximately 1-2 hours to complete. Four hours following the initial exercise session, I will perform a *Subsequent Exercise Performance Test*, consisting of 30-min of moderate-intensity cycling followed by a 20 km time-trial, which will take approximately 40-45 min to complete. I will be encouraged to treat the trial as a competitive event, and give my best effort throughout the trials. Twenty-four hours after the trial, I will return to the laboratory to assess markers of muscle damage and recovery.

I will complete the following procedures at various time-points during the protocol:

Blood draws – Blood will be collected immediately prior to exercise, between the two exercise sessions, and 24-hours following exercise. Each of these blood draws will be conducted by inserting a needle into a vein in the upper forearm. During each sample, small amounts of blood (~5-10 ml) will be obtained, and utilized to measure either insulin or markers of muscle damage. Some blood from these collections will also be used to assess genotype variations that may be related to muscle damage. In addition, blood glucose and lactate will be assessed once during each exercise session, using a finger-stick technique, in which a very small amount of blood (~0.5 ml) is obtained. The total amount of blood obtained during each trial period is approximately 20 ml, (60 ml over the course of the entire trial). This total amount is less than 1/5 of a can of soda, or 15% of the amount given when donating blood in a single session (approximately 1 pint, or 473 ml). The indicated frequency of blood draws represents the bare minimum number required to be able to assess the dependent measurements indicated within this protocol (muscle damage, insulin, glucose & lactate). Study participants should refrain from donating blood during the study period.

Measurements During Exercise:

At one time-point during the *high-intensity aerobic intervals*, and also once during the *Subsequent Exercise Performance Test*, the following measurements will be obtained:

Metabolic Measurements. I will have metabolic measurements, such as oxygen uptake, ventilation, etc. measured using a SensorMedics metabolic cart. To do this, I will breathe through a mouthpiece/breathing apparatus that collects my expired breath for approximately 5 minutes. The mouthpiece/breathing apparatus will NOT be worn during exercise other than at the indicated time-point.

Ratings of Perceived Exertion –I will provide subjective ratings of my exertion level. I will do this by pointing to my corresponding level of exertion (rated numerically from 6-20) on a Borg RPE scale.

Heart Rate. I will have my heart rate measured using a Polar heart rate monitor that is worn around my chest throughout each exercise session.

Measurements Before and After Exercise:

Muscle Soreness and Fatigue Ratings. I will provide muscle soreness and fatigue ratings using a series of 100mm visual analog scales, with 0 indicating (i.e.) no muscle soreness and 100 indicating impaired movement due to muscle soreness. These ratings will be obtained immediately prior to the first exercise session (*high-intensity aerobic intervals*), immediately prior to the second exercise session (*Subsequent Exercise Performance Test*), and 24-hours following the first exercise session.

Muscle Function - I will perform a maximal strength test of my quadriceps at the following time-points: prior to the first exercise session (*high-intensity aerobic intervals*), following the first exercise session, prior to the second exercise session (*Subsequent Exercise Performance Test*), following the second exercise session, and 24-hours following the first exercise session.

During this test, I will be seated in a modified chair, and asked to push as hard as possible against a shin pad that will be connected to a force transducer for about 5 repetitions with a minute rest in between.

Sports Beverages and Dietary Controls: I will consume 625 ml of a sports beverage at the following time-points: a) immediately following the first exercise session (*high-intensity aerobic intervals*), b) two-hours following the first exercise session, and c) immediately following the second exercise session (*Subsequent Exercise Performance Test*). I will perform the study protocols on three occasions (separated by 7-14 days each), using a different sports beverage on each occasion. Treatments will contain the same amount of fluid but will contain differing amounts of other nutrients (carbohydrates, fat and/or protein). The specific composition of beverages utilized in the study will not be revealed to me during the study. However, I may request this information upon completion of the study.

Two hours prior to each trial, I will receive and consume a small standardized meal. I may select the specific food type from one of three choices, but the specific foods consumed during this period will be replicated across each of the three treatment periods. In addition, I will be encouraged to maintain consistent dietary and exercise habits across the duration of the two treatment periods. I will complete a dietary log of all foods consumed during the days prior and following each of the exercise sessions. In addition, I will record the type, amount and intensity of all exercise performed during these same time periods. I will also refrain from heavy exercise for 48-hours prior to each exercise test, and will not exercise between the exercise tests and follow-up testing (24 hours).

Risks:

Participants are expected to be honest about disclosing all known risk factors to the researcher. There are no known risk factors associated with sports beverage consumption. The beverages may contain milk ingredients, so participants with known milk allergies will be excluded from study participation.

According to the American College of Sports Medicine, the risks associated with maximal exercise/testing for healthy individuals are very minimal. Any subjects who do not meet the criteria for "low risk" will not be allowed to participate in the study. In the unlikely event of cardiac or other complications during exercise, an emergency plan is in place. This includes immediate access to a phone to call emergency personnel. In addition, each of the investigators is CPR certified.

The exercise protocol may result in minor-moderate levels of muscle soreness and fatigue for 1-2 days following each exercise session. However, the level of muscle soreness is expected to be lower than levels normally experienced when people perform other 'normal' activities that are not part of their regular exercise routine (i.e. if a cyclist played a game of basketball with friends for 2 hours).

The risks of blood draws include possible mild bruising, and the risk of transfer of blood-borne pathogens. This risk is considered to be very minimal, and all safety precautions for handling blood samples will be followed according to OSHA protocols. The investigators have been trained in phlebotomy and completed JMU blood-borne pathogen training. The total amount of blood removed in these trials is considered very minimal. Approximately 60-70 ml of blood collected during the entire study, which is considered very minimal. For reference, this is less than 1/5 of a can of soda, or 15% of the amount given when donating blood in a single session (approximately 1 pint, or 473 ml).

Benefits:

Benefits include free maximal oxygen uptake testing, use of cutting-edge nutrition products, and a \$200 stipend for study completion. In the event that I am unable to complete all testing, payments will be distributed in the following manner: \$25 for completing the familiarization trial, \$25 for completing experimental trial 1, and \$50 for completing experimental trial 2.

Inquiries:

If you have any questions or concerns, please contact Dr. Michael Saunders at saundemj@jmu.edu or (540) 568-8121. In the case of any immediate concerns or adverse reactions during the study, contact Dr. Saunders at his cell phone (540) 560-3032.

Confidentiality:

All data and results will be kept confidential. Subjects will be assigned an identification code. At no time will a subject's name be identified with individual data. The researcher retains the right to use and publish non-identifiable data. All data will be kept secured in a locked cabinet. Upon completion of the study, all information that matches up individual respondents with their answers will be destroyed. Final aggregate results will be made available to participants upon request.

Freedom of Consent

Your participation is entirely voluntary. You are free to choose not to participate. Should you choose to participate, you can withdraw at any time without consequences of any kind.

I have read this consent form and I understand what is being requested of me as a participant in this study. I freely consent to participate. I have been given satisfactory answers to my questions. The investigator provided me with a copy of this form. I certify that I am at least 18 years of age.

 Name of Subject (Printed)

 Name of Researcher (Printed)

 Name of Subject (Signed)

 Name of Researcher (Signed)

 Date

 Date

For questions about your rights as a research subject, you may contact the chair of JMU's Institutional Review Board (IRB). Dr. David Cockley, (540) 568-2834, cocklede@jmu.edu.

Appendix II

AHA/ACSM Health/Fitness Facility Pre-participation Screening Questionnaire

Assess your health status by marking all *true* statements

History

You have had:

- ☐ a heart attack
- ☐ heart surgery
- ☐ cardiac catheterization
- ☐ coronary angioplasty (PTCA)
- ☐ pacemaker/implantable cardiac
- ☐ defibrillator/rhythm disturbance
- ☐ heart valve disease
- ☐ heart failure
- ☐ heart transplantation
- ☐ congenital heart disease

If you marked any of these statements in this section, consult your physician or other appropriate health care provider before engaging in exercise. You may need to use a facility with a ***medically qualified staff***.

Symptoms

- ☐ You experience chest discomfort with exertion
- ☐ You experience unreasonable breathlessness
- ☐ You experience dizziness, fainting, or blackouts
- ☐ You take heart medications

Other Health Issues

- ☐ You have diabetes
- ☐ You have asthma or other lung disease
- ☐ You have burning or cramping sensation in your lower legs when walking short distances
- ☐ You have musculoskeletal problems that limit your physical activity
- ☐ You have concerns about the safety of exercise
- ☐ You take prescription medication(s)

Cardiovascular risk factors

- ☐ You are a man older than 45 years
- ☐ You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal
- ☐ You smoke, or quit smoking within the previous 6 months
- ☐ Your blood pressure is > 140/90 mmHg
- ☐ You do not know your blood pressure
- ☐ You take blood pressure medication
- ☐ Your blood cholesterol level is > 200 mg/dl
- ☐ You do not know your cholesterol level
- ☐ You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister)
- ☐ You are physically inactive (i.e. you get < 30 minutes of physical activity on at least 3 days of the week)
- ☐ You are > 20 pounds overweight

If you marked two or more of the statements in this section, you should consult your physician or other appropriate health care provider before engaging in exercise. You might benefit from using a facility with a ***professionally qualified exercise staff*** to guide your exercise program.

- ☐ None of the above

You should be able to exercise safely without consulting your physician or other appropriate health care provider in a self-guided program or almost any facility that meets your exercise program needs.

Appendix III

Borg Rating of Perceived Exertion Scale (6 - 20)

Exertion	RPE
no exertion at all	6
extremely light	7
	8
very light	9
	10
light	11
	12
somewhat hard	13
	14
hard (heavy)	15
	16
very hard	17
	18
extremely hard	19
maximal exertion	20

Appendix IV

Muscle Soreness Questionnaire

Subject #____ **Trial #**____ **Circle: Pre 4-Post 24-Post** **Date:**_____

Muscle Soreness

➔ Please place a mark on the line below corresponding to your level of muscle soreness

0 millimeters (left) = complete absence of muscular soreness

100 millimeters (right) = extremely sore with noticeable pain and stiffness at all times

0 mm _____ 100 mm

Score: _____mm (to be completed by researcher)

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